The Role of Alpha v Beta 6 Integrin in Enamel Biomineralization

by

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Abstract

Tooth enamel has the highest degree of biomineralization of all vertebrate hard tissues. During the secretory stage of enamel formation, ameloblasts deposit an extracellular matrix that is in direct contact with ameloblast plasma membrane. Although it is known that integrins mediate cell-matrix adhesion and regulate cell signaling in most cell types, the receptors that regulate ameloblast adhesion and matrix production are not well characterized. Thus, we hypothesized that αvβ6 integrin is expressed in ameloblasts where it regulates biomineralization of enamel. Human and mouse ameloblasts were found to express both β6 integrin mRNA and protein. The maxillary incisors of *Itgb6-/-* mice lacked yellow pigment and their mandibular incisors appeared chalky and rounded. Molars of *Itgb6-/-* mice showed signs of reduced mineralization and severe attrition. The mineral-to-protein ratio in the incisors was significantly reduced in *Itgb6-/-* enamel, mimicking hypomineralized amelogenesis imperfecta. Interestingly, amelogenin-rich extracellular matrix abnormally accumulated between the ameloblast layer of *Itgb6-/-* mouse incisors and the forming enamel surface, and also between ameloblasts. This accumulation was related to increased synthesis of amelogenin, rather than to reduced removal of the matrix proteins. This was confirmed in cultured ameloblast-like cells, which did not use αvβ6 integrin as an endocytosis receptor for amelogenins, although it participated in cell adhesion on this matrix indirectly via endogenously produced matrix proteins. In summary, integrin αvβ6 is expressed by ameloblasts and it plays a crucial role in regulating amelogenin deposition/turnover and subsequent enamel biomineralization.

Preface

A version of chapter 2 has been published. Mohazab L, Koivisto L, Jiang G, Kytömäki L, Haapasalo M, Owen GR, Wiebe C, Xie Y, Heikinheimo K, Yoshida T, Smith CE, Heino J, Häkkinen L, McKee MD, and Larjava H. Critical role for $\alpha v\beta 6$ integrin in enamel biomineralization. Journal of Cell Science. 2013; 126: 732-744. The research question of chapter 2 was identified and project was designed by Dr. Hannu Larjava. Leila Mohazab collected and analysed most of the data under the guidance of Dr. Hannu Larjava, and participated in writing the manuscript. L. Koivisto assisted in western blotting and cell spreading experiments and manuscript writing; G. Jiang performed real-time PCR analyses; L. Kytömäki performed and analyzed the gene arrays; M. Haapasalo and C. Wiebe. assisted in collecting samples for immunohistochemistry; G.R. Owen assisted in endocytosis experiments; Y. Xie assisted in immunolocalization studies; K.H. Heikinheimo provided samples and assisted in manuscript writing; T. Yoshida performed in situ hybridization experiments; C.E. Smith analyzed the mineral and protein contents of enamel; J. Heino assisted in gene profiling and manuscript writing; L. Häkkinen participated in experiment planning and manuscript writing; M.D. McKee performed hard tissue sectioning and electron microscopy studies and assisted in manuscript writing; H. Larjava supervised all experiments and participated in manuscript writing.

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Acronyms

αvβ6 alpha-v-beta-6.

AI Amelogenesis imperfecta.

AMBN Ameloblastin.

AMELX Amelogenin gene on chromosome X.

AMELY Amelogenin gene on chromosome Y.

ANK Ankylosis gene.

DEJ Dentinoenamel junction.

DLX3 Distal-less homeobox 3.

ECM Extracellular matrix.

EMD Emdogain.

ENAM Enamelin.

ERM Epithelial cells rests of Malassez.

FAM20A Family with sequence similarity 20, member A.

FAM20B Family with sequence similarity 20, member B.

FAM20C Family with sequence similarity 20, member C.

FAM83H Family with sequence similarity 83, member H.

H&E Hematoxylin and eosin.

HERS Hertwigs epithelial root sheath.

IgG Immunoglobulin G.

IRC Indirect resin crown.

Itgb4 β4 integrin.

Itgb6 β6 integrin.

Itgb6-/- β6 integrin knockout.

KLK4 Kallikrein-4.

LAP Latency associated peptide.

LLC Large latent complex.

LTBP Latent TGF-β binding protein.

MMP20 Matrix metallopeptidase20 (enamelysin).

ODAM Odontogenic ameloblast-associated.

RGD Argenine-glycine-aspartic acid.

SDS-PAGE Sodium dodecyl sulfate polyacrylamide gels.

SLC Small latent complex.

SSC Stainless steel crown.

TGFβRI TGF-β type I receptor.

TGFβRII TGF-β type II receptor.

TGF-β Transforming growth factor- β.

TGF-β1 Transforming growth factor- β1.

TPA Tetradecanoylphorbol-13-acetate.

WDR72 WD repeat-containing protein 72.

WT Wild-type.

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Most of all I am very grateful to my brother, Dr. Ali Reza Mohazab, for his tremendous technical help in various steps.

To my family, thank you for your constant love and support, and for believing in me.

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Dedication

This work is dedicated to my loving family and in particular to my brother, Dr. Ali Reza Mohazab. Thank you for your endless support and encouragement.

Chapter 1

Review of the literature

1.1 Introduction

Enamel is the hardest mineralized tissue in the body and the only calcified tissue that is produced by epithelial-derived cells, called ameloblasts. It covers the crowns of teeth and protects them from functional wear and decay. Amelogenesis, or enamel development, consists of secretory, transition and maturation stages. During the secretory stage, ameloblasts actively secrete enamel proteins such as amelogenin (the most abundant enamel matrix protein) (Eastoe 1979), ameloblastin (Krebsbach et al. 1996), and enamelin (Hu et al. 1997) into the enamel matrix. This extracellular matrix undergoes enzymatic modification by enamelysin (MMP20) and kallikrein-4 (KLK4) in the transition and maturation stages. This results in the formation of a mature enamel that is mainly composed of hydroxyapatite crystallites and a minor amount of proteins (Bartlett et al. 1996; Nanci and Smith 2000). Several hereditary conditions affecting enamel have been described. In certain cases, such as amelogenesis imperfecta, in which enamel formation and mineralization is affected, the enamel defects are caused by mutations in amelogenin, enamelin, MMP20, or KLK4 (Hu et al. 2007). The defective enamel mineralizations leads to extensive wear and decay in both the primary and permanent dentition. As a result, these patients may lose their teeth at a young age or require extensive restorative procedures to prevent further decay and/or attrition (Crawford et al. 2007).

Not all cases of defective enamel biomineralization can be explained by the above gene defects, suggesting that also other mechanisms are involved. During amelogenesis, ameloblast plasma membrane has direct contact with the matrix and the developing enamel crystals (Nanci and Smith 2000). The receptors of ameloblasts that mediate ameloblast-matrix adhesion, matrix organization, and signaling are not well characterized, but may play an important role in enamel biomineralization. Integrins mediate cell-matrix adhesion and signaling in most cell types (Hynes 2004). The secretome of rat incisor enamel organ has been reported to include the $\beta 6$ integrin transcript, but nothing is known about $\alpha v \beta 6$ integrin in enamel formation (Moffatt et

al. 2006). In the present study, we have investigated the expression and function of $\alpha v\beta 6$ integrin during mouse enamel formation. The aims of our study were

- To characterize enamel defects in detail in $\beta 6$ integrin-null animals
- To uncover the molecular mechanisms by which $\beta 6$ integrin deficiency affects enamel biomineralization

Our hypotheses were

- $\alpha v\beta 6$ integrin is expressed in a specific developmental stage in ameloblasts and that lack of its expression severely disturbs enamel mineralization.
- $\alpha v\beta 6$ integrin causes cell signaling via endogenous TGF-$\beta 1$ activation which regulates the enamel matrix production and processing required for biomineralization.

1.2 Tooth development

Teeth develop from oral ectoderm and mesenchyme, and this interaction between the epithelial tissue and its underlying mesenchymal tissue is under strict genetic control (Thesleff and Hurmerinta 1981; Thesleff 2006). Morphogenesis and cell differentiation in the tooth germ are regulated by epithelial-mesenchymal signaling, which consists of a chain of sequential and reciprocal events (Thesleff et al. 1995; Thesleff 2003). Experiments have shown that in the very early stages of tooth formation, the epithelium induces the mesenchyme to acquire odontogenic potential (Thesleff et al. 1995). In the later stages however, signals from the ectomesenchyme can elicit tooth formation from a variety of epithelia, even non-dental epithelium. (Thesleff et al. 1995). During tooth development, the ectomesenchymal cells, which originate from neural crest cells, and the epithelial cells slowly acquire higher levels of differentiation and become odontoblasts and ameloblasts respectively (Thesleff and Hurmerinta 1981). The epithelial derived ameloblast cells are responsible for the formation of highly mineralized, acellular enamel, while the odontoblasts make the more resilient, vital dentin. In fact, other than the enamel (and some cementum), the rest of the tooth tissues as well as its supporting tissue are all derived directly from neural crest cells.

Tooth development has been divided into three overlapping periods of initiation, morphogenesis, and cell differentiation (Figure 1.1.; Kollar 1978;

Thesleff and Hurmerinta 1981). During this process, a complex cascade of gene expression takes place and results in oral ectoderm thickening, budding, growth, folding, and formation of tooth crown (Thesleff 2003).

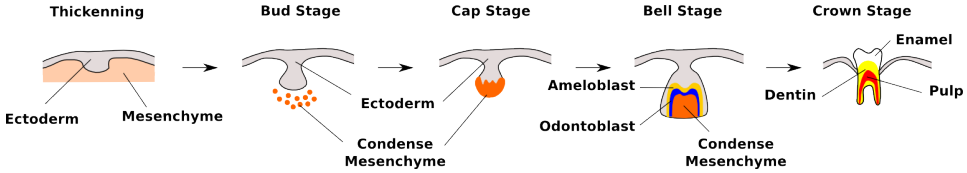

Figure 1.1: Tooth formation stages. Schematic representation shows the different stages of tooth formation. There is a constant reciprocal interaction between the epithelial and mesenchymal tissue during tooth formation.

During initiation, the primary epithelial band forms as a continuous thick band of epithelium in both the maxilla and mandible, from which the dental lamina and vestibular lamina are formed (Thesleff and Hurmerinta 1981). The cells forming the vestibular lamina initially enlarge, but then degenerate in order to form a cleft that becomes the future vestibule. The dental lamina is formed from epithelial outgrowths into the underlying mesenchyme and corresponds to the position of future teeth row (Kollar 1978). Furthermore, localized thickenings within the primary epithelial band gives rise to placodes, which bud into the underlying mesenchymal cells and function as one of the first signalling centres of the tooth (Pispa and Thesleff 2003). It is hypothesized that one entire tooth family (incisor, canine, or molar) is formed from one dental placode (Thesleff 2003). Signaling molecules such as FGFs (fibroblast growth factor), BMPs (bone morphogenic proteins, belonging to the TGF-β superfamily) and Wnts regulate the formation of placodes (Thesleff 2003). Placodal signals in turn, regulate the budding of epithelium and the mesenchymal condensation (Thesleff 2003).

The morphogenesis phase encompasses the bud, cap, and bell stages of tooth formation, where the size and shape of the tooth is established by morphogenic movements of the tissue (Thesleff and Hurmerinta 1981). During the bud stage, there is an epithelium incursion into the mesenchyme, which is accompanied by the condensation of the mesenchymal cells surrounding the budding epithelium (Thesleff et al. 1995). This ball of condensed ectomesenchymal cells gives rise to two clusters of cells: the dental papilla and the dental follicle (Thesleff 2003). The dental papilla will form the dentin and the pulp, while the dental follicle gives rise to the cementoblasts and the periodontal tissue (Tummers and Thesleff 2008). At this stage, the dental epithelium also has two distinct cell lineages which consist of peripheral

basal cells and the centrally located stellate reticulum cells (Tummers and Thesleff 2008).

During the cap and bell stages, there is further morphogenesis of the epithelium and the crown morphology becomes apparent as the epithelium grows and surrounds the dental papilla (Thesleff et al. 1995). It is during the transition from bud to cap stage that morphologic differences between tooth germs gives rise to different types of teeth. At the cap stage, the epithelial bud continues to proliferate into the mesenchyme, and surrounds the dental papilla (Thesleff 2003). During transition to this stage, clusters of non-dividing epithelial cells give rise to the enamel knot, which expresses genes for many signaling molecules, such as Shh (sonic hedgehog) BMPs, FGFs, and Wnts (Jernvall and Thesleff 2000; Thesleff 2003). Each tooth has a single primary enamel knot at the cap stage. Signals from the enamel knots regulate growth and cuspal morphogenesis by controlling the initiation of secondary enamel knots (Jernvall and Thesleff 2000; Thesleff 2003). Secondary enamel knots express most of the same signaling molecules and appear at the sites of epithelial foldings, corresponding to the tips of the future cups in the molar teeth (Jernvall and Thesleff 2000). While signals from the enamel knot affect the epithelial and mesenchymal tissues, the reciprocal interaction between the epithelium and mesenchyme are needed to maintain the enamel knot (Thesleff 2003).

The final shape of the crown is established at the bell stage. During this stage, cell differentiation and mineralization takes place as the epithelial and mesenchymal cells differentiate into ameloblasts and odontoblasts respectively, and these cells start to deposit the enamel and dentin matrices (Thesleff 2003).

Moreover, it is during the cap and bell stages that an epithelial outgrowth on top of the ectomesenchyme forms the enamel organ. The cells at the periphery of enamel organ, facing the dental follicle, form the outer enamel epithelium, and the cells that border the dental papilla form the inner enamel epithelium (Tummers and Thesleff 2003). The inner enamel epithelium is responsible for the formation of the enamel. The outer and inner enamel epithelia are continuous, and the region where they meet is called the cervical loop (Tummers and Thesleff 2003). Stratum reticulum cells and a layer of stratum intermedium cells facing the inner enamel epithelium occupy the core of the loop (Tummers and Thesleff 2003). The cervical loop is responsible for the continuous cell division, until the crown reaches its full size, after which, the cells give rise to the epithelial component of root formation. While the cervical loop is maintained in continuously growing teeth such as the rodent incisor, in humans it undergoes a structural

modification upon root formation (Tummers and Thesleff 2003).

After crown formation is completed, the stellate reticulum and stratum intermedium cells are lost, leaving only a double layer of basal epithelium known as Hertwig's epithelial root sheath (HERS) (Tummers and Thesleff 2003; Tummers and Thesleff 2008). The sheath grows down and encompasses all, but the basal portion of dental pulp. It directs root growth and shape, and results in dentin formation in the root by initiating the differentiation of odontoblasts from the ectomesenchymal cells in the outer dental papilla (Tummers and Thesleff 2003; Tummers and Thesleff 2008). Cementogenesis in the root occurs when HERS degrades and gives rise to epithelial cells covering the root, known as the epithelial cells rests of Malassez (ERM) (Tummers and Thesleff 2008). Through the network lining of ERM, dental follicular cells migrate and contact the newly formed dentin (Tummers and Thesleff 2008). This results in the dental follicle cells to differentiate into cementoblasts depositing the cementum (Tummers and Thesleff 2008).

The mouse incisor grows continuously and is functionally and morphologically subdivided into two domains: the labial crown analog and the lingual root analog (Figure 1.2; Tummers and Thesleff 2008). The labial side is covered by enamel produced by ameloblasts, and the lingual side is covered by dentin and cementum deposited by odontoblasts and cementoblsats (Tummers and Thesleff 2008). The cervical loop with a core of stellate reticulum cells resides in the base of the incisor, and thus both the crown and root analog are generated continuously by the apical end of the incisor (Harada et al. 2002; Tummers and Thesleff 2008). It has been suggested that the progeny of dividing stem cells from the core of the loop integrate into the basal layer of epithelium, proliferate to form transit amplifying cells around the loop, and then differentiate into ameloblasts or root epithelium depending on their regulatory environment (Tummers and Thesleff 2008). Signaling molecules from the mesenchyme, such as FGF-3, FGF-10, BMP-4 and Activin were found to modulate and regulate the proliferation and maintenance of the epithelial stem cell progeny (Harada et al. 2002; Wang et al. 2007). Lastly, Follistatin, which is a TGF-β antagonist, has been identified as the key signaling molecule that inhibits ameloblast differentiation and enamel deposition on the lingual side of the incisor (Wang et al. 2007).

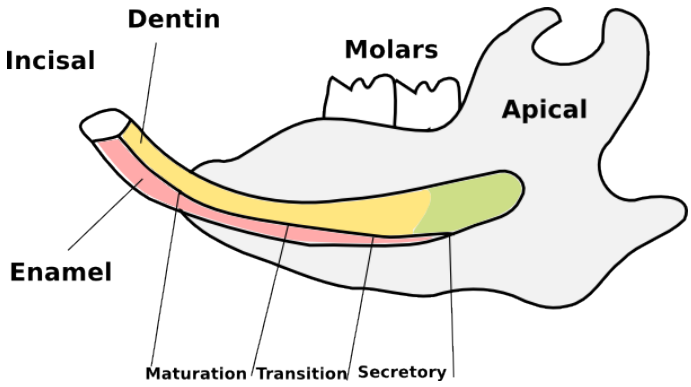

Figure 1.2: Mouse hemi-mandible. The mouse incisor grows continuously and is covered by enamel only on its labial side. The different stages of amelogenesis can be found along the labial length of the incisor.

1.3 Amelogenesis

Enamel is the most highly mineralized tissue in the body. It is unique from other mineralized tissues because it is noncollagenous and derived from ameloblast cells, which are of epithelial origin (Fincham Moradian-Oldak and Simmer 1999). At the time of tooth eruption ameloblast cells are lost, and therefore, mature enamel is acellular. Due to this absence of ameloblasts, mature enamel cannot renew itself and does not undergo remodeling. (Fincham Moradian-Oldak and Simmer 1999). Mature enamel is approximately ninety-five percent mineral, four percent water, and one percent organic matter (Deakins and Volker 1941; LeFevre and Manly 1932). The inorganic portion of enamel is long, thin closely packed crystals, composed of calcium hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ (Simmer and Hu 2001; Elliott Holcomb and Young 1985; Young 1974). The hydroxyapatites group together to form the fundamental organizational units of enamel: enamel rods and interrods. The crystals in the rods are parallel to the long axis of the rod, while the crystals in the interrods run in different directions (Simmer and Fincham 1995). The cylindrical enamel rods are surrounded by interrod enamel, and run from the dentinoenamel junction (DEJ) to the tooth surface. The narrow space that forms the boundary between the rod and interrod enamel contains organic material and is known as the rod sheath. The proteins ameloblastin and amelogenin are major components of the rod sheath (Hu et al. 2007). Rodents do not have a well defined rod sheath in their enamel (Uchida et al. 1998).

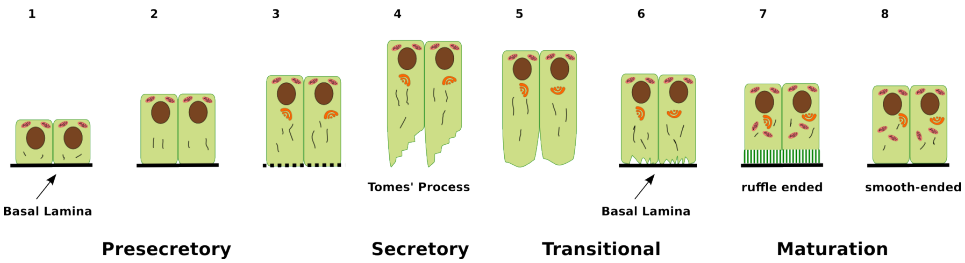

Figure 1.3: Ameloblasts' morphological changes during amelogenesis. At first the cells of the inner enamel epithelium rest on the basement membrane (1). These ameloblasts then elongate and differentiate into pre-secretory ameloblasts (2). The pre-secretory ameloblasts degenerate the basement membrane, as they start secreting the enamel proteins (3). The secretory ameloblasts form Tomes' processes, which are projections along the face of the ameloblast that organizes the enamel crystals (4). Once the enamel achieves its full thickness, the secretory stage ends and the ameloblasts lose their Tomes' process (5). In the transition stage, the ameloblasts decrease their height, a new basement membrane is deposited, and the cells start to produce enzymes to degrade the accumulated protein matrix (6). During the maturation stage, the ameloblasts alter between the ruffle-ended (7) and smooth-ended phases (8). This modulation between the two phases is essential for the mineralization of the enamel.

Amelogenesis or enamel formation is a highly specialized, multi-stage process that takes place in a unique extracellular matrix produced by ameloblast cells. Amelogenesis occurs in three stages: presecretory, secretory and maturation. During these stages, an organic matrix is secreted, the crystals are nucleated and elongated, the organic material and water and subsequently lost, and the enamel crystals mature (Reith 1970). Throughout this process, ameloblast cells go through progressive morphological changes (Figure 1.3; Pindborg and Weinmann 1959).

Following the initiation of dentinogenesis in the developing tooth, cells of the inner enamel epithelium resting on a basement membrane (basal lamina), differentiate into pre-secretory ameloblasts. These ameloblast cells elongate, become polarized, prepare for protein synthesis, and lose the ability to undergo mitosis (Simmer and Fincham 1995). The preameloblasts begin secreting enamel proteins followed by degeneration of the basement membrane (Simmer and Hu 2001). Degradation of the basement membrane, which originally serves to separate preameloblasts from preodontoblasts, is

required for enamel formation to occur (Hu et al. 2007). In addition, loss of this basement membrane marks the initiation of dentin mineralization (Hu et al. 2007). As the basal lamina disintegrates, the presecretory ameloblasts send cytoplasmic processes trough the gaps and continue the secretion of enamel proteins on the irregular mineralizing surface of the dentin (Hu et al. 2007; Ronnholm 1962a; Ronnholm 1962b). The secreted enamel matrix mineralizes into a smooth thin layer of aprismatic, rodless enamel, and is perforated by odontoblast processes (Ronnholm 1962b). Thus, the dentin and enamel are linked and the dentinoenamel enamel junction is established.

During the secretory stage, the ameloblasts migrate away from the DEJ. As they do this, they continue to produce and lay down enamel proteins into the space that was previously the basal lamina, on top of the existing enamel crystallites (Hu et al. 2007). The secretory ameloblasts are tall polarized columnar cells (Lacruz et al 2010). They are linked by cell-cell junctions and also further develop secretory structures called the Tomes' processes (Kallenbach 1973; Reith and Boyde 1978; Ronnholm 1962a; Ronnholm 1962b). Tomes' processes are cytoplasmic extensions that extend in the newly formed enamel, and organize the enamel crystals into rod and interrod enamel (Risnes 1998). Secretions from the proximal part of the Tomes' process, which contacts the adjacent ameloblast, form the interrod enamel, while secretions from the distal portion of the processes, which interdigitates into the enamel, form the rod enamel. During this stage, the ameloblasts secrete proteins at a mineralization front and the enamel crystals begin to elongate (Hu et al. 2007). The newly laid down enamel matrix is immediately, but very lightly, mineralized and the mineralization front retreats with the Tomes' process as the enamel crystals grow in length (Hu et al. 2007). Each enamel rod is synthesized by the Tomes' process of one ameloblast cell (Hu et al. 2007). The enamel crystals elongate in increments, with each increment representing the amount of crystal growth in one day (Hu et al. 2007; Simmer and Hu 2001). The amount of enamel deposition per day varies depending on the systemic factors (Simmer and Hu 2001). At the end of the secretory stage, the enamel is partially mineralized, and the crystallites achieve their full length, corresponding to the final thickness of the enamel (Hu et al. 2007; Simmer and Fincham 1995).

In the maturation stage, the principal function of ameloblasts is to remove water and organic material from the enamel matrix, and supply it with calcium and phosphate ions (Simmer and Fincham 1995). During this stage, the enamel crystallites grow in width and thickness and the enamel hardens (Simmer and Hu 2001). In order for the mineralization of enamel to occur, the ameloblasts must undergo a transition that involves a reduc-

tion in their secretory activity. These ameloblasts decreases in height and lose their Tomes' processes (Smith 1979). In addition, they begin secreting kallikrein-4 (KLK4), a protease that degrades the accumulated proteins (Hu et al. 2007). During the maturation stage, ameloblasts modulate between ruffle and smooth-ended morphologies (Josephsen and Fejerskov 1977). Ruffle-ended ameloblasts are bounded by tight junctions and have considerable endocytotic activity (Lacruz et al. 2010; Simmer and Fincham 1995; Takano 1995). They participate in the active transport of calcium and phosphate into the enamel matrix (Lacruz et al. 2010; Simmer and Fincham 1995; Takano 1995). As the enamel proteins are degraded and removed, the sides of the enamel crystals are exposed, and active incorporation of mineral ions into crystal takes place by ruffle ended ameloblasts (Hu et al. 2007). Furthermore, the ruffle-ended ameloblasts produce bicarbonate ions, which alkalinizes the matrix and prevents demineralization of the crystals (Lacruz et al. 2010; Simmer and Fincham 1995; Smith 1998). In contrast, smooth ended ameloblasts have no endocytotic activity, and do not have distal tight junctions (Simmer 1995, Lacruz 2010). A lack of the tight junctions in these cells permits the exit of large molecules and protein fragment to the enamel surface, from between the cells (Lacruz et al 2010; Simmer and Fincham 1995). The alteration between the ruffle-ended and smooth-ended ameloblasts in the maturation stage is therefore essential in enamel formation. Ultimately, these cells function to create and maintain an environment where the pH is tightly regulated, and the removal of organic matrix and deposition of mineral content can take place (Lacruz et al. 2010).

Moreover, during the maturation stage, the ameloblasts also deposit a unique basal lamina, to which they are attached via hemidesmosomes (Dos Santos Neves et al. 2012). This basal lamina is highly glycosylated and contains laminin-5 (Dos Santos Neves et al. 2012). This basal lamina is responsible for the attachment of ameloblasts to the enamel, regulating the movement of material into and out of the enamel matrix, and relaying information about the status of enamel formation to the ameloblast cells (Dos Santos Neves et al. 2012). Amelotin and odontogenic ameloblast-associated (ODAM) are components of the basal lamina, which are secreted by the maturation stage ameloblasts (Dos Santos Neves et al. 2012; Moffatt et al. 2006).

In continuously erupting rodent incisors, the different stages of amelogenesis are found in series along the length of a single tooth. Enamel formation occurs sequentially from the apical end to the incisal end of the incisor, and distinct zones of secreting, maturing, and mature enamel are found on the

tooth (Smith and Nanci 1989).

1.4 Enamel proteins

During amelogenesis, ameloblasts secrete a variety of enamel proteins and proteinases that are involved in enamel formation. Amelogenenin, ameloblastin, enamelin are the major structural proteins, while enamelysin (MMP20) and kallikrein-4 (KLK4) are the major proteinases.

Amelogenin is secreted during the secretory stage of amelogenesis and plays a role in the regulation of enamel crystal pattern and thickness (Gibson et al. 2001). It is the most abundant enamel protein, comprising about eighty to ninety percent of total enamel proteins (Deutsch 1989; Fincham Moradian-Oldak and Simmer 1999; Hu et al. 2007). Ninety percent of the human amelogenin protein is expressed from genes located on the X-chromosome (AMELX), and ten percent are expressed from the Y-chromosome (AMELY) (Lau et al. 1989; Nakahori Takenaka and Nakagome 1991; Salido et al. 1992). Amelogenin is secreted in a variety of isoforms, but the major isoform has a molecular weight of approximately 25 kDa (Hu et al. 2007; Simmer and Hu 2001). The alternative splicing of the amelogenin transcript, as well as post-translational modifications such as proteolysis, result in a range of protein weights for amelogenin, as seen on a sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) analysis (Fincham et al. 1991; Gibson et al. 1991; Salido et al. 1992). Amelogenin is critical for normal development of enamel, due to its role in the inhibition of lateral growth of hydroxyapatite crystals. The different amelogenin isoforms self-assemble into structures known as nanospheres (Fincham et al. 1994). Nanospheres are responsible for regulating crystal spacing, and thus determine the width and thickness of the of enamel crystals (Fincham et al. 1995). Furthermore, amelogenin contains histidine, which causes the absorption of hydrogen ions, and therefore functions to regulate the pH of the enamel matrix (Simmer and Hu 2001).

Ameloblastin (AMBN), also known as amelin and sheathlin, is mainly secreted during the secretory stage of enamel formation. It comprises about five to ten percent of enamel proteins, and has a molecular weight of 65 to 70 kDa (Yamakoshi et al. 2001). Its gene loci are located on chromosome 4q in humans (MacDougall et al. 1997) and on chromosome 5 in mice (Krebsbach et al. 1996). Two ameloblastin isoforms are secreted due to alternative splicing of mRNA transcript (Hu et al. 1997), and the protein is proteolytically cleaved at its N-terminal soon after its secretion. The N-terminal

cleavage product is accumulated in the enamel sheath space, separating rod and interrod enamel (Uchida et al. 1995). Amelobastin is a critical cell adhesion molecule which aids in the adherence of ameloblasts to the enamel surface (Fukumoto et al. 2004; Fukumoto et al 2005). Ameloblastin also plays a crucial role in maintaining ameloblast differentiation, which makes it essential for normal enamel formation (Fukumoto et al. 2004; Fukumoto et al. 2005). It binds to the ameloblast cell, inhibits its proliferation, and maintains the differentiated phenotype of secretory ameloblasts (Fukumoto et al. 2004; Fukumoto et al. 2005).

Enamelin (ENAM) is the largest and least abundant of the enamel matrix proteins. It has a molecular mass of roughly 200 kDa (Hu et al 1997; Hu et al. 2000) and comprises about three to five percent of the structural proteins in developing enamel. Enamelin gene is located on chromosome 4q, near the ameloblastin gene (Hu et al. 2000). Enamelin is secreted during the secretory stage, and is rapidly cleaved by proteinases upon its release. Its function is to promote crystal nucleation and elongation (Hu and Yamakoshi 2003).

Proteolysis, carried out by metalloproteinases and serine proteinases, is essential for enamel biomineralization (Hart et al. 2004; Kim et al. 2005). These proteases are expressed at different times and have different functions. Proteolytic activities cleave enamel proteins and produce stable cleavage products by changing the structure and properties of the protein. Moreover, proteolysis results in degradation of the extracellular organic matrix in the maturation stage, and thus allows crystal growth and mineralization (Lu et al. 2008; Smith 1998).

MMP20 is a metalloproteinase that is predominantly expressed during the secretory and early maturation stages of amelogenesis (Bartlett et al. 1996; Hu et al. 2002). It is located on chromosome 11 (Llano et al. 1997) and migrates as a double band at 40 and 45 kDa on SDS-PAGE (Fukae et al. 1998; Smith et al. 1996). MMP20 selectively cleaves enamel proteins and slowly degrades them, and thus allows crystallites to grow in width and thickness (Lu et al. 2008). It predominantly cleaves amelogenin (Nagano et al. 2009) and ameloblastin (Chun et al. 2010) during the secretory stage. Uncleaved enamel proteins and cleaved products of proteins segregate into different compartments within the enamel layer, which suggests that they might have different functions (Simmer and Hu 2002).

KLK4 is a serine protease that shows up as two bands at 31 and 34 kDa on SDS-PAGE (Tanabe 1984). The gene is located on chromosome 19 (DuPont et al. 1999; Hu et al. 2000) and the protein is secreted during the maturation stage of amelogenesis. KLK4 aggressively removes the remaining

extracellular organic matrix, following the termination of enamel protein secretion, and makes way for hardening of the enamel. Residual amelogenin in the developing enamel comprises the main substrate for the KLK4 enzyme (Ryu et al. 2002). It also degrades glycosylated enamelin cleavage product that is not digested by MMP20 (Yamakoshi et al. 2006). Interestingly, KLK4 does not degrade the enamel proteins that take part in making the basal lamina during the maturation stage (Takano 1979).

1.5 Amelogenesis imperfecta

1.5.1 Overview of amelogenesis imperfecta

Amelogenesis imperfecta (AI) represents a collection of genetic disorders that affects enamel formation in primary as well as permanent dentition in the absence of systemic manifestations (Aldred and Crawford 1995; Witkop 1988). Clinically, teeth may present as being discolored and sensitive with rough texture, pits, or grooves (Crawford et al. 2007). Moreover, these patients are prone to periodontal conditions, predominantly gingivitis, and may show dental anomalies such as reduced crown size and taurodontism (Poulsen et al. 2008). Patients with AI lose their teeth at a young age or require extensive restorative procedures to prevent further decay and/or attrition. Furthermore, it has profound psychological effects such as poor self-esteem (Hu et al. 2007).

The clinical manifestation is enamel that is either hypoplastic, hypomature/ hypomineralized, hypocalcified or a combination of these, depending on the timing of the enamel formation defect (Stephanopoulos et al. 2005; Witkop and Sauk 1976; Wright et al. 2009). Hypoplastic AI results in enamel that is pathologically thin and rough textured with a yellowish-brown color; it forms if the defects occur in the secretory stage of amelogenesis and results in crystal elongation being disrupted; (Hu and Yamakoshi 2003). On the other hand, hypomineralized/hypomature AI results in enamel that is pathologically soft, opaque and brownish colored; it forms if the defect occurs in the maturation stage of amelogenesis and affects the removal of organic matrix (Hu and Yamakoshi 2003). In this case, it is important to note that the teeth are normal in size (Hu and Yamakoshi 2003). Lastly, hypocalcified AI results from the defects occurring during the mineralization process and contains enamel that is very soft with a rough texture and wears away quickly (Hu and Yamakoshi 2003).

The disorder's mode of inheritance is either autosomal-dominant, autosomal-recessive, or X-linked (Aldred et al. 2003; Backman 1997; Crawford et al.

2007). This is a genetically heterogeneous disorder and to date, mutations in the following candidate genes have been found to cause different types of AI: amelogenin (AMELX), enamelin (ENAM), enamelysin (MMP20), kallikrein-4 (KLK4), family with sequence similarity 83, member H (FAM83H), Family with sequence similarity 20, member A (FAM20A), WD repeat-containing protein 72 (WDR72), and distal-less homeobox 3 (DLX3) (Crawford et al. 2007; Wright et al. 2011; Lee et al. 2011). Interestingly, mutations in the genes that encode the enamel extra-cellular matrix proteins and proteases account for only one quarter of all AI cases, and nearly half of the AI causes are of an unknown etiology (Hart et al. 2003b; Kim et al. 2006; Lee et al. 2011). The prevalence of AI ranges from 1 in 700 in Sweden (Backman and Holm 1986) to 1 in 14000 in the USA (Witkop 1957). AI has been classified into 14 clinical subtypes based on mode of inheritence and enamel phenotype (Witkop 1988; Aldred and Crawford 1995).

Numerous mouse models have been generated that have one or a combination of the candidate genes altered and/or knocked-out, and thus provide tools for understanding AI pathogenesis (discussed below).

1.5.2 Mutations in the amelogenin gene (AMELX)

Amelogenin is the most abundant enamel matrix protein and its function is thought to be the regulation of crystal growth and the organization of enamel rods during amelogenesis (Gibson et al. 2001). The amelogenin gene resides on the sex chromosomes and consists of seven exons and six introns (Brookes et al. 1995; Lau et al 1989). Although in humans the amelogenin gene is located on both the X chromosome (AMELX) and the Y chromosome (AMELY), only 10% of the amelogenin mRNA expression is from AMELY (Lau et al. 1989; Nakahori et al. 1991; Salido et al. 1992). X-linked AI is a phenotypically and genotypically diverse disorder, which results from mutations in the Xp22.1-p22.3 chromosome of AMELX (Hart et al. 2000; Lagerstrom et al. 1991; Lagerstrom-fermer et al. 1995; Wright et al. 2003). To date, there are 18 known AMELX mutations (Hu et al. 2012) that result in distinct and variable enamel phenotypes depending on the type and location of the mutation in the gene (Stephanopoulos et al. 2005; Wright et al. 2003). In humans, the phenotypical appearance ranges from hypomineralized/hypomature enamel to hypoplastic enamel (Hart et al. 2000). Moreover, X-linked AI affects males and females quite differently. Males have a single copy of the X chromosome therefore the enamel is severely affected because all alleles are affected if there is a mutation, whereas females are less severely affected by a single mutation because they

have two X chromosomes. (Crawford and Aldred 1992). Affected females have a distinctive phenotype that consists of alternating vertical ridges of normal and defective enamel, which results from alternative inactivation of either one of the X chromosomes, be it the normal or the mutated, in different cohorts of enamel-forming cells (Hu et al. 2007). This is known as "lyonization" (Hu et al. 2007).

In general, AMELX mutations consist of signal peptide, and mutations that causes a total loss of amelogenin protein, missense mutations affecting the N-terminal region specifically, and mutations affecting the C-terminal region in specific (Crawford et al 2007).

Five AMELX signal peptide mutations have been reported that all result in the formation of very thin, hypoplastic enamel (Kida et al. 2007; Kim et al. 2004; Lagerstrom-fermer et al. 1995; Sekiguchi et al. 2001b). These include a deletion of nine nucleotides in exon 2 that replace amino acids 5 through 8 with threonine (g.14_22del) (Lagerstrom-fermer et al. 1995), a single base substitution in exon 2 (g.11G>A) that introduces a premature stop codon (Sekiguchi et al. 2001b), two missense mutations in exon 2 (g.2T>C and g.11G>C) that affect the translation initiation codon and/or the secretion of amelogenin (Kim et al. 2004), and lastly a missense point mutation in exon 5 (g.3458C>G) (Kida et al. 2007). With the g.3458C>G mutation lyonization is observed as the affected females show only vertical ridges on normal-sized teeth, whereas the males have a generalized thin, discolored enamel (Kida et al. 2007).

There are four mutations that affect the N-terminus of AMELX (Wright et al. 2003). One involves a single nucleotide substitution in exon 5 (g.3458delC), and a subsequent introduction of a premature stop codon, which results in a hypomineralized/hypomature enamel accompanied with some degrees of hypoplasia (Aldred et al. 1992; Lench et al. 1994). The other three mutations in the N-terminus are single nucleotide substitutions that result in single amino acid changes leading to formation of a brown discolored enamel (Collier et al. 1997; Hart et al. 2002; Lench and Winter 1995; Ravassipour et al. 2000). While one involves exon 5 (g.3455C>T) and results in hypomineralized/hypomature enamel (Lench and Winter 1995), the other two mutations occur in exon 6 (g.3781C>A and g.3803A>T) and produce a hypomature enamel (Collier et al. 1997; Hart et al. 2002; Ravassipour et al. 2000).

There are six mutations that affect the C-terminus of AMELX by introducing a premature stop codon in exon 6 and clinically present as smooth hypoplastic enamel (g.4046delC, g.4114delC, g.3993delC, g.3958delC, g.4144G>T, and g.4090delC) (Greene et al. 2002; Hart et al. 2002; Kindelan et al. 2000;

Lench and Winter 1995; Sekiguchi et al. 2001a; Lee et al. 2011). Five of these mutations involve single deletions, while the fifth mutation involves a single nucleotide change (Stephanopoulos et al. 2005; Lee et al. 2011).

Another form of X-linked AI results from a 5 Kb deletion from exon 3 to exon 7 that knocks out the AMELX gene (g.1148-54del) and causes a combined phenotype of hypomineralization and hypomaturation of the enamel (Lagerstrom et al. 1991).

Lastly, a partial deletion of protein ARHGAP6 that causes the removal of all AMELX, has also been shown to cause X-linked AI (Hu et al. 2012). ARHGAP6 is a GTPase-activating protein of the Rho-GAP family, which is expressed in different tissues at low levels and regulates actin polymerization (Prakash et al. 2000). The AMELX gene is situated within the first intron of ARHGAP6 (Crampton et al. 2006). Partial deletions of ARHGAP6 which cause the removal of all AMELX gene results in hypoplastic AI, in which the enamel is thin and rough (Hu et al. 2012).

Table 1.1 summarizes the different AMELX mutations and their respective genotypes and phenotypes.

In summary, signal peptide mutations and mutations in the C-terminus of the protein result in hypoplastic forms of X-linked AI, while mutations in the N-terminus are associated with hypomineralized/hypomature X-linked AI (Kang et al. 2009; Lee et al. 2011). Despite the number of mutations that have been identified, only 5% of families with AI show an X-linked pattern (Backman and Holmgren 1988).

Amelogenin knockout mice display thin hypoplastic enamel, which is disorganized due to a total loss of prism structure (Gibson et al. 2001). Moreover, their incisors and molars fracture frequently, and there is a marked chalky-white discoloration of the incisors (Gibson et al. 2001). This amelogenin null phenotype indicates that amelogenins are indeed responsible for the organization of crystal pattern and play a role in the regulation of enamel thickness (Gibson et al. 2001).

Table 1.1: AMELX mutations.

Location	Protein	Gene	Inheritance	Phenotype	References
Intron 1	ARHGAP6	g.302534_ 398773del96240	X-linked	Hypoplastic	Hu et al. (2012)
Exon 2	ARHGAP6	g.363924_ 416577del52654insA	X-linked	Hypoplastic	Hu et al. (2012)
Exon 2	p.I5_A8delinsT	g.14_22del	X-linked	Hypoplastic	Lagerstrom-Fermer et al. (1995)
Exon 2	p.W4X	g.11G>A	X-linked	Hypoplastic	Sekiguchi et al. (2001)
Exon 2	p.M1T	g.2T>C	X-linked	Hypoplastic	Kim et al. (2004)
Exon 2	p.W4S	g.11G>C	X-linked	Hypoplastic	Kim et al. (2004)
Exon 5	p.52R	g.3458C>G	X-linked	Hypoplastic	Kida et al. (2007)
Exon 5	p.P52fsX53	g.3458delC	X-linked	Hypomineralized/ Hypomature	Aldred et al. (1992) Lench et al. (1994)
Exon 5	p.T51I	g.3455C>T	X-linked	Hypomineralized/ Hypomature	Lench and Winter (1995)
Exon 6	p.70T	g.3781C>A	X-linked	Hypomature	Collier et al. (1997) Hart et al. (2000) Ravassipour et al. (2000)
Exon 6	p.77L	g.3803A>T	X-linked	Hypomature	Hart et al. (2002)
Exon 6	p.P158HfsX187	g.4046delC	X-linked	Smooth hypoplastic	Lench and Winter (1995)
Exon 6	p.L181CfsX187	g.4114delC	X-linked	Smooth hypoplastic	Kindelan et al. (2000) Hart et al. (2002)
Exon 6	p.Y147fsX187	g.3993delC	X-linked	Smooth hypoplastic	Greene et al. (2002)
Exon 6	p.H129fsX187	g.3958delC	X-linked	Smooth hypoplastic	Sekiguchi et al. (2001)
Exon 6	p.E191X	g.4144G>T	X-linked	Smooth hypoplastic	Lench and Winter (1995)
Exon 6	p.P173LfsX16	g.4090delC	X-linked	Hypoplastic	Lee et al. (2011)
Exon 3- Exon 7	p.18del	g.1148_54del	X-linked	Hypomineralized/ Hypomature	Lagerstrom et al. (1991)

1.5.3 Mutations in the enamelin gene (ENAM)

Enamelin, the largest and the least abundant enamel protein, plays a role in enamel mineralization and crystal elongation (Chan et al. 2010; Hu et al. 2000; Hu et al. 2007). Human enamelin is localized on chromosome 4 (4q11-q21) and consists of nine exons and eight introns (Crawford et al. 2007; Dong et al. 2000; Hu et al. 2000; Hu et al 2001; Rajpar et al. 2001). There are 12 known ENAM mutations and the associated phenotype ranges from minor localized hypoplastic enamel to severe hypoplastic enamel (Chan et al. 2010; Simmer et al. 2012).

Most ENAM mutations cause an autosomal-dominant hypoplastic form of AI. The first ENAM mutation identified consists of a G to A transition in intron 8 (g.6395G>A), which causes a deletion of exon 8 and a subsequent formation of a severe form of autosomal-dominant smooth hypoplastic AI (Rajpar et al. 2001). A clinical manifestation of small, yellow teeth with little or no enamel layer is observed (Rajpar et al. 2001). An upstream translation termination codon in exon 5 (g.2382A>T) and a splice accepter site mutation in intron 6 (g.4806A>C) both result in formation of autosomal-dominant local hypoplastic AI (a milder form of AI), in which, patients have sensitive teeth and show localized enamel pits and horizontal grooves (Kim et al. 2005a; Kim et al. 2006; Mardh et al 2002). A missense mutation in exon 10 (g.12663C>A), which introduces a premature stop codon and truncates the enamelin protein after 246 amino acids, also results in formation of autosomal-dominant localized enamel hypoplasia (Ozdemir et al. 2005a). A splice donor site mutation of a single-G deletion at the end of exon 9 and the beginning of intron 9 (g.8344delG) results in autosomal-dominant smooth hypoplastic AI (Hart et al. 2003a; Kida et al 2002; Kim et al. 2005a; Pavlic et al. 2007). These patients have yellow colored teeth that are hypersensitive to cold stimuli and a thin enamel layer with a surface texture that varies from smooth to rough with or without horizontal grooves (Hart et al. 2003a; Kida et al 2002; Kim et al. 2005a; Pavlic et al. 2007). A substitution of guanine with thymine in exon 9 (c.G817T) also results in autosomal-dominant hypoplastic AI with a manifestation of severe generalized hypoplastic enamel (Gutierrez et al. 2007). Lastly, a novel heterozygous in exon 4 of ENAM has recently been identified, which causes a frameshift in the coding region of the signal peptide and results in hypoplastic AI (Simmer et al. 2013).

Other ENAM mutations result in dose-dependent associated phenotypes (Chan et al. 2010; Hart et al. 2003; Ozdemir et al. 2005a; Pavlic et al 2007). A 2bp (AG) insertion mutation in exon 10 of ENAM 4q13.3

(g.13185/6insAG) introduces a premature termination codon and results in the formation of hypoplastic enamel (Chan et al. 2010; Hart et al. 2003; Kang et al. 2009; Ozdemir et al. 2005a; Pavlic et al. 2007). While severe generalized hypoplastic defects with an anterior open bite is inherited in an autosomal recessive pattern in the homozygous individuals, localized hypoplastic enamel pittings (with or without open bite) with a chalky white colored enamel is inherited in an autosomal dominant pattern in the heterozygous members (Chan et al. 2010; Hart et al. 2003; Ozdemir et al. 2005a; Pavlic et al. 2007). In fact, some heterozygous members of this mutation show no detectable enamel defect at all (Kang et al. 2009). Compound heterozygotes for the mentioned mutation (the AG insertion in exon 10) and a novel insertion mutation of seven amino acids (g.12946_12947insAGTCAGTACCAGTACTGTGTC) also display a severe generalized hypoplastic autosomal-recessive AI (Ozdemir et al. 2005a). A single T deletion in exon 10 (g.14917delT) results in a premature termination codon and the subsequent formation of hypoplastic enamel in an autosomal-dominant pattern (Kang et al. 2009). The heterozygote individuals for this mutation also display a dose-dependent phenotype ranging from chalky white enamel with mild localized pitting to prominent horizontal grooves and hypoplasia (Kang et al. 2009). Lastly a missense ENAM mutation (g.12573C>T) that replaces leucine for a phosphorylated serine results in the formation mildly hypoplastic enamel and only localized pitting in heterozygotes, but severe hypoplasia and enamel malformations are observed in homozygote individuals (Chan et al. 2010). These findings indicate that ENAM mutations may result in dose-dependent enamel phenotypes; generalized hypoplastic AI segregating as a recessive trait and localized enamel pitting segregating as a dominant trait (Chan et al. 2010; Ozdemir et al. 2005a). Table 1.2 summarizes the different ENAM mutations and their respective genotypes and phenotypes.

Similar to the AI cases in humans, enamelin defects in mice is also dose-dependent and thus heterozygous enamelin knockout ($Enam^{+/-}$) and homozygous enamelin knockout ($Enam^{-/-}$) mice possess very different phenotypes (Hu et al. 2008). The $Enam^{+/-}$ mice have relatively mild defects in general and have maxillary incisors that phenotypically range from having a smooth, brownish yellow colored enamel to a chalky white colored enamel (Hu et al. 2008). While the maxillary incisors are somewhat similar to the wild-type mice, the mandibular incisors are rough, and blunt, with a chalky white colored enamel that has lost its translucency (Hu et al. 2008). Although some enamel abrasion is evident in the molar cusps, the molars in these mice are normal for the most part (Hu et al. 2008).

On the other hand, enamel abnormalities are evident on all the teeth in the Enam$^{-/-}$ mice. In these animals, both the maxillary and the mandibular incisors show rough and broken surfaces with a white opaque appearance, while the molar cusps display severe coronal wear (Hu et al. 2008). Furthermore, the less the amount of enamelin present, the less mineralization that takes place. As such, enamel proteins are accumulated in the extracellular space of ameloblasts in Enam$^{-/-}$ mice without any mineralization taking place (Hu et al. 2008). In fact, these mice lack true enamel altogether, and their teeth are rather covered by a thin layer of mineral that is formed by a completely different mechanism (Hu et al. 2008).

Mice that have a mutated enamelin gene display similar defects than those observed in the ENAM knockout mice. The homozygous mutated mice show a complete loss of enamel on the incisors and molars, while the heterozygous mice had rough and cracked enamel surfaces (Masuya et al. 2005; Seedorf et al. 2004; Seedorf et al. 2007). Overall, enamelin mutated mice have a similar phenotype as the hypoplastic forms of AI (Masuya et al. 2005; Seedorf et al. 2004; Seedorf et al. 2007). The defects displayed by enamelin mutated mice and knockout mice emphasize the critical role that enamelin plays in enamel crystal elongation and mineralization.

Table 1.2: ENAM mutations

Location	Protein	Gene	Inheritance	Phenotype	References
Exon 4	p.Asn36Ilefs56	g.2979delA	AD	Local Hypoplastic	Simmer et al. (2013)
Exon 5	p.K53X	g:2382A>T	AD	Local hypoplastic	Mardh et al. (2002) Kim et al. (2006)
Intron 6	p.M71_Q157del	g.4806A>C	AD	Local Hypoplastic	Kim et al. (2005)
Intron 8	p.A158_Q178del	g.6395G>A	AD	Smooth hypoplastic	Rajpar et al. (2001)
Exon 9	p.R179M	c.G817T	AD	Hypoplastic	Gutierrez et al. (2007)
Intron 9	p.N197fsX277	g.8344delG	AD	Smooth hypoplastic	Kida et al. (2002) Hart et al. (2003) Kim et al. (2005) Pavlic et al. (2007)
Exon 10	p.S216L	g.12573C>T	AR; AD-localized enamel pitting	Generalized hypoplastic (recessive trait); Localized enamel pittings (dominant trait)	Chan et al. (2010)
Exon 10	p.S246X	g.12663C>A	AD	Local hypoplastic	Ozdemir et al. (2005)
Exon 10	p.V340-M341insSQYQYCV	g.12946_12947insAGTCAGTACCAGTACTGTGTC	AR;AD-localized enamel pitting	Generalized hypoplastic (recessive trait); Localized enamel pittings (dominant trait)	Ozdemir et al. (2005)
Exon 10	p.P422fsX448	g.13185/6insAG	AR;AD-localized enamel pitting	Generalized hypoplastic (recessive trait); Localized enamel pittings (dominant trait)	Hart et al. (2003) Ozdemir et al. (2005) Pavlic et al. (2007) Kang et al. (2009) Chan et al. (2010)
Exon 10	p.P998fsX1062	g.14917delT	AD	Hypoplastic	Kang et al. (2009)
Not mentioned in the study	p.R179-N196del	g.9045A>G	AD	Hypoplastic	Wright et al.(2011)

1.5.4 Mutations in the enamelysin gene (MMP20)

MMP20 is a tooth specific gene that is located on chromosome 11 (11q22.3-q23) (Llano et al. 1997) and is comprised of ten exons and nine introns (Caterina et al. 2002). This proteinase is expressed by ameloblasts during the secretory and early maturation stages of amelogenesis and plays a role in removing enamel matrix proteins (Hu et al. 2002). To date, three mutations have been identified in the enamelysin gene, all of which result in an autosomal recessive form of hypomatured AI (Kim et al. 2005b; Ozdemir et al. 2005b; Papagerakis et al. 2008). The first is a point mutation in intron 6 (g.IVS6S-2A>T) that destroys the splice acceptor and results in the formation of a hypomature enamel (Kim et al. 2005b). The second MMP20 mutation involves a single base mutation in exon 5 (g.16250T>A) that alters the amino acid histidine to glutamine (Ozdemir et al. 2005b). Individuals homozygous for these two types of MMP20 mutations have an anterior open bite with discolored, pigmented, opaque teeth; the teeth have a rough surface and are very brittle, even though they are of normal thickness (Kim et al. 2005b; Ozdemir et al. 2005b). This is typically the phenotype that is associated with autosomal recessive pigmented hypomaturation AI (Kim et al. 2005b; Ozdemir et al. 2005b). The third point mutation takes place in exon 1 of MMP20 (g.102G>A) and introduces a premature stop codon which causes autosomal recessive hypoplastic-hypomature AI (Papagerakis et al. 2008). These individuals have very thin, hypomineralized enamel that has yellowish pigmentation and chips away easily (Papagerakis et al. 2008).

MMP20 null mice display a severe AI phenotype (Caterina et al. 2002). These mice do not process amelogenin properly, have an altered enamel matrix and rod pattern, display severe attrition of the molars, and have hypoplastic enamel that does not adhere properly to dentin (Caterina et al. 2002). Furthermore, unlike the wild-type and heterozygous animals, the enamel matrix in the MMP20 null mice is not removed nor processed in the secretory stage of enamel development; in fact the enamel matrix persists even in the late maturation stage of amelogenesis (Caterina et al. 2002). Lastly, the enamel mineral in the MMP20 null mice is reduced by 50% and its hardness is decreased significantly compared to the wild-type mice (Bartlett et al. 2004). This mouse model indicates that MMP20 is essential for processing and removal of the enamel matrix proteins, especially amelogenins, in the secretory and maturation stages of amelogenesis; overall, a lack of this proteinase has profound effects on enamel development (Bartlett et al 2004; Caterina et al. 2002). Similar to heterozygous humans, heterozygous mice have a normal phenotype, thus suggesting that one func-

tional MMP20 allele is sufficient for normal enamel formation (Caterina et al. 2002; Ozdemir et al. 2005b).

1.5.5 Mutations in the kallikrein-4 gene (KLK4)

KLK4 gene is on chromosome 19 (19q13.3-13.4) and consists of six exons and five introns (DuPont et al. 1999; Hu et al. 2000). Its expression starts during the transition and maturation stages of enamel formation and continues through tooth eruption where it is responsible for further degradation of amelogenin cleavage products (Hu et al. 2002). Although the expression of KLK4 and MMP20 are temporally different, mutation in KLK4 also causes autosomal recessive pigmented hypomaturation AI with a similar phenotype as the AI caused by a mutated MMP20 gene (Hart et al. 2004; Wright et al. 2006). KLK4 mutation occurs in exon 4 (G.2142G>A) and results in a truncated KLK4 that lacks S207 of the catalytic triad, which is essential for the protein's proteolytic activity (Hart et al. 2004). Similar to the first two types of MMP20 mutations mentioned, KLK4 mutation result in an enamel with a normal thickness and prismatic architecture (Hart et al 2004; Wright et al. 2006). However, the enamel formed is discolored, incompletely mineralized, and has an increased protein content (Hart et al. 2004; Wright et al. 2006).

As for the KLK4 knockout mice, heterozygous and homozygous mice develop quite different phenotypes. While the tooth shape, size and color of heterozygous mice are very similar to the wild-type, the homozygous mice posses chalky white enamel that easily chips away and is abraded following weaning (Simmer et al. 2009). Although the enamel crystallites of KLK4 null mice do not fully mature, the enamel layer achieves normal thickness and width (Simmer et al. 2009). Furthermore, lack of KLK4 results in a high retention of enamel proteins which act as a physical barrier and block the expansion and maturation of crystals (Simmer et al. 2009). In this case, individual enamel crystallites fail to grow together and interlock (Simmer et al. 2009). The observations from human cases and mouse experiments show that KLK4 is critical for removal of enamel proteins, crystallite growth and enamel mineralization; however, it does not play a major role in enamel thickness (Hart et al 2004; Simmer et al. 2009; Wright et al. 2006).

1.5.6 Mutations in the family with sequence similarity 83, member H gene (FAM83H)

FAM83H is located on chromosome 8q24.3 and while its function is not well known yet, the protein appears to be associated with intracellular vesicles and trans Golgi organelle (Ding et al. 2009). Interestingly, the FAM83H is one of the two genes (other one being WDR72) that plays a role in the etiology of AI which does not encode for a secreted protein (Ding et al. 2009). Although FAM83H is expressed in developing teeth, its expression is not confined to teeth and its role in amelogenesis is unknown (Kweon et al. 2013; Lee et al. 2009). Furthermore, even though the protein is expressed in many other tissues throughout the body, no defects in these tissues have been identifies in the AI patients (Kweon et al. 2013).

Mutations in FAM83H are the only known cause of autosomal dominant hypocalcified AI, and account for the highest percentage and most severe cases of AI than any other gene mutations (Lee et al. 2011). All of the AI-causing mutations in FAM83H thus far have been either frameshift or non-sense mutations in the last coding exon (exon5), which results in a truncated protein (Kim et al. 2008; Lee et al. 2008; Hart et al. 2009; Wright et al. 2009; El-Sayed et al. 2010; Lee et al. 2011). While most of the mutations result in a hypocalcified enamel covering the whole crown, the more down-stream mutations result in a localized phenotype confined to the cervical half of the crown only (Wright et al. 2009). To date, 16 novel mutations have been identified, all of which cause premature translation termination between the amino acids Ser^{287} and Glu^{694} (Lee et al. 2011).

Mice that overexpress FAM83H gene have shown to not have any enamel defects (Kweon et al. 2013). Further animal research is needed to shed light on the role of this gene in amelogenesis.

1.5.7 Mutations in the family with sequence similarity 20, member A gene (FAM20A)

Mutations in the family with sequence similarity 20, member A gene is yet another novel mutation that plays a role in the etiology of AI. Family with sequence 20 (FAM20) has three members: FAM20A, FAM20B, and FAM20C (Nalbant et al. 2005). FAM20 protein family plays different roles in mineralized tissue. FAM20B is a kinase implicated in phosphorylation and control of proteoglycans, and it is expressed during the maturation stage of amelogenesis (Koike et al. 2009; O'Sullivan et al. 2011). FAM20C has been shown to be an essential gene for normal bone development (Hao

et al. 2007; Simpson et al. 2007; Wang et al. 2010; Ishikawa et al. 2012). Although the exact function of FAM20A is yet to be determined, the gene is located on chromosome 17q24.2 and it is expressed in the enamel organ and the gingiva (Cho et al. 2012; O'Sullivan et al. 2011). Mutations in the FAM20A gene causes hypoplastic AI and gingival overgrowth (Cho et al. 2012, O'Sullivan et al. 2011). Furthermore, prolonged retention of primary teeth, intrapulpal calcificaition and delayed eruption of permanent teeth are some other features that are found in these AI patient (O'Sullivan et al. 2011).

1.5.8 Mutations in the WD repeat-containing protein 72 gene (WDR72)

Recently, novel mutations in the WDR72 gene have been identified as a significant cause of autosomal recessive hypomaturation AI (El-Sayed et al. 2009; El-Sayed et al. 2011). WDR72 is an intracellular protein with a β propeller structure, and plays a role in protein-protein interactions; however, its exact function is unknown for the most part (El-Sayed et al. 2009; El-Sayed et al. 2011). A point mutation in exon 15 of WDR72 (c.2348C>G) results in a premature stop codon, causing hypomaturation AI (El-Sayed et al. 2009; El-Sayed et al. 2011). Although the mechanism by which this mutation results in hypomaturation AI is not known, it is suggested that the late stage of enamel maturation is affected (El-Sayed et al. 2009; El-Sayed et al 2011). More research and animal experiments are needed to understand the role of WDR72 in amelogenesis.

1.5.9 Mutations in the distal-less homeobox 3 gene (DLX3)

The DLX3 gene is a homeodomain transcriptional factor that is expressed in dental epithelium and mesenchyme, as well as in other locations such as differentiating epidermal cells, neural crest, hair follicles, and placenta (Beanan and Sargent 2000). This factor behaves as a transcriptional activator and is located on chromosome 17 (q21 – q22) (Dong et al. 2005). A two base pair deletion in this gene results in a frameshift alteration and introduces a premature stop codon that truncates the protein by 88 amino acids (Dong et al. 2005). This results in the formation of a unique kind of syndromic AI known as AI hypomaturation-hypoplasia type with taurodontism (AIHHT) (Dong et al. 2005). This is inherited in an autosomal dominant pattern and manifests as thin, hard enamel with an enlarged pulp chamber (Dong et al. 2005). Diagnosis of AIHHT should not be confused with tricho-dento-

osseous (TDO) syndrome, which is also an autosomal dominant syndrome. TDO syndrome is caused by a four base pair deletion in DLX3 and manifests as kinky, curly hair at birth, enlarged pulp chambers, enamel defects, and usually is accompanied by craniofacial abnormalities (Beanan and Sargent 2000; Price et al. 1998).

In mouse development, DLX3 is essential for embryonic survival and as such the early lethality of the DLX3 knockout mice makes it very difficult to study the enamel formation later in development of these mice (Beanan and Sargent 2000).

1.6 Dental management of patients with AI

Dental management of AI patients is very complex in both the functional and esthetic aspects. In the past, patients with AI were treated with extractions and the construction of complete removable dentures (Gokce et al. 2007). Recently however, bonded porcelain inlays, stainless steel crowns, metal-ceramic crowns, and adhesive plastic restorations are used more commonly as means of treatment. The treatment plan for an AI patient must take into consideration many factors such as the patient's age and socioeconomic status, the type of AI and the severity of the disorder, and the patient's intraoral condition (Akin et al. 2007).

Pediatric dentists play a crucial role in the early diagnosis and management of AI. The goal is to maintain the health of the primary teeth as much as possible, and to closely monitor the development of the permanent teeth (Ng and Messer 2009). When the patient has mixed dentition, the treatment's aim is to improve esthetics, reduce dental hypersensitivity and attrition, maintain the vertical dimension, and restore the masticatory function (Pires dos Santos et al. 2008). As such, the treatment of AI starts in childhood and is based on early prevention and intervention (Crawford et al. 2007), which includes meticulous oral hygiene and protection of teeth with different types of crowns.

Maintaining good oral hygiene and reducing the risk of getting dental caries are very important, as poor dental and gingival health further complicate the restorative management of the teeth (Sapir and Shapira 2007). The child's diet should be modified to minimize taking cariogenic foods, which contain a high amount of sugar, stick to the tooth, and are erosive. Oral hygiene instructions should include proper toothbrush and brushing method, and the use desensitizing toothpaste (Sapir and Shapira 2007). Furthermore, topical use of fluoride, and daily use of sodium fluoride rinse, and

casein phosphopeptide-amorphous calcium phosphate must be implemented early (Ng and Messer 2009). Together, these help to resist demineralization, decrease tooth sensitivity, reduce caries risk, and enhance enamel remineralization (Ng and Messer, 2009; Sapir and Shapira 2007). Lastly, the patient should regularly receive professional tooth cleaning and calculus removal to improve periodontal health (Ng and Messer 2009).

Full coverage restorations are considered to be one of the most effective ways of managing tooth sensitivity and poor esthetics in children (Ng and Messer 2009). Stainless steel crowns (SSC) are often used to cover the primary and permanent molars, as early as possible (Ng and Messer 2009; Pires dos Santos et al. 2008; Sapir and Shapira 2007). SSCs maintain the vertical dimension of occlusion, conserve tooth vitality, integrity and prevent posteruptive breakdown, manage tooth sensitivity, and establish correct interproximal and occlusal relationship (Ng and Messer 2009; Sapir and Shapira 2007). SSCs are also quick and easy to place, which is an advantage particularly when managing children (Ng and Messer 2009), and are not as costly or technique sensitive as cast restorations (Sapir and Shapira 2007). However, SSCs require quite a bit of tooth preparation and need to be replaced with cast restorations later on (Ng and Messer 2009).

As for the anterior primary teeth, crown restoration with resin composite or laminate veneers may be used (Ng and Messer 2009). Indirect resin crown (IRC) is suggested by some authors as an optimal intermediary treatment option for AI patients with mixed dentition (Quinonez et al. 2000). IRCs are very cost effective and provide very good esthetics for children (Quinonez et al. 2000). Moreover, with the continued tooth eruption, IRCs can be easily modified at the gingival margin by adding composite resin to the exposed margin (Quinonez et al. 2000).

Treating the permanent dentition in an AI patient is more complex and requires a multidisciplinary approach which includes periodontic, orthodontics, endodnotic, and prosthodontic treatments (Ng and Messer 2009). Orthodontic treatment can help to close spaces between teeth and fix the malocclusion, or to separate teeth before crown placement in order to conserve tooth material in permanent teeth (Sapir and Shapira 2007). However, retention of orthodontic brackets to teeth may be a problem in some AI patients (Sapir and Shapira 2007). In some cases, a headgear is fabricated to be worn at night time in order to fix the occlusion (Sapir and Shapira 2007).

Simple microabrasions (mostly in cases of hypomaturation AI), gold or stainless steel crowns, all ceramic crowns, metal-ceramic crowns, porcelain laminate veneers, porcelain onlays, direct resin composite restorations, indi-

rect resin composite laminate veneers, and indirect resin composite partial or full crowns are some of the treatment options that may be used to improve esthetics and/or restore teeth with posteruptive breakdown (Yamaguti et al. 2006). Gold based alloys are considered a good choice because they are quite wear resistant and also cause minimal wear of the opposing enamel (Ng and Messer 2009; Yamaguti et al. 2006). However, they have an unnatural appearance and should be used for the posterior teeth mostly (Yamaguti et al. 2006). Metal-ceramic crowns on the other hand provide a more appealing esthetics, but they are abrasive to the opposing tooth enamel and as such, this limits their use in AI patients who have a fragile enamel to begin with (Yamaguti et al. 2006). All ceramic crowns are not as strong as metal crowns, have a brittle characteristic, and also wear down the opposing enamel (Yamaguti et al. 2006). However, they provide great esthetics and therefore they may be used as an anterior teeth restoration (Ng and Messer 2009). Porcelain laminate veneers are also a popular choice for the treatment of anterior teeth. Unlike complete crown restoration that is an invasive procedure with a substantial amount of tooth removal, veneers provide the same optimal esthetics with a very conservative tooth preparation (Yamaguti et al. 2006). Veneers are however not a good choice if the patient suffers from tooth sensitivity (Ng and Messer 2009).

Recently, the use resin composite restoration associated with glass ionomer cements has increased (Sabatini and Guzman-Armstrong 2009; Yamaguti et al. 2006). Resin composite restorations provide excellent esthetics and tooth preservation; however, bonding resin composite to AI-affected enamel could be an issue. The bond strength between the resin composite and hypomineralized enamel is severely compromised compared to normal enamel (Sapir and Shapira 2007). The bond between the enamel and the restoration greatly depends on the enamel surface changes after acid etching, and some studies have shown that phosphoric acid that is commonly used to etch the enamel may cause more enamel loss than the self-etching primers (Seow and Amaratunge 1998; Sapir and Shapira 2007). Therefore, self-etching primers may be used as an alternate means of preparing the tooth for the resin composite bond (Sapir and Shapira 2007; Sabatini and Guzman-Armstrong 2009). Self-etching primers are also recommended to use in AI patients because they are simple to use, require few steps and less time, and cause less postoperative sensitivity (Sapir and Shapira 2007). Furthermore, some self-etching primers have fluoride releasing properties and an antibacterial component, both of which are advantageous to AI patients (Sapir and Shapira 2007). The overall literature however, does suggest that typical etch patterns are produced in most variants of the AI, except in smooth hypoplastic

AI (Seow and Amaratunge 1998), and therefore bonding of resin composite should be feasible in most of the AI patients. Moreover, some authors suggest that pre-treating the enamel with 5% sodium hypochlorite removes the excess non-mineralized acid insoluble proteins from teeth, and significantly enhances the bond strength (Seow and Amaratunge 1998; Ng and Messer 2009). It is also essential to remove all clinically defective, soft enamel in order to obtain a stronger resin bond to the potentially underlying normal enamel (Sabatini and Guzman-Armstrong 2009). The disadvantage of composite resins is its undesirable properties such as microleakage, staining, low abrasion resistance, and plaque accumulation (Saha and Saha 2011).

In conclusion, the dental management of AI patients is very complex and case specific. It starts with proper diagnosis and early intervention in childhood. The treatment includes many disciplines and modalities, and requires follow-up and maintenance to achieve long-term success. Moreover, these patients require substantial emotional support, especially in the cases of children and adolescents.

1.7 Other mutations in mice that affect the enamel formation

Aside from the discussed genes that play a role in the etiology of AI in humans, there are a number of other genes that play pivotal roles in amelogenesis, and their mutations cause various enamel defects in transgenic mice. Table 1.3 which is adapted from a review paper shows a list of enamel defects that are caused by different mutations in transgenic mice (Bei 2009). These different genes are responsible for cell-signalling, cell-cell adhesion, and transcription factors which affect the amelogenesis process. Below, a few examples of the selected genes that result in formation of a defective enamel are discussed.

Cell signalling molecules are essential endogenous factors that play a role in ameloblast cell differentiation (Bei 2009). TGF-β1 is a cell signalling molecule which is a member of the TGF-β super-family. It is expressed during early development in different tissues and cells, including the ameloblast cell (Chai et al. 1994; Chai et al. 1999; Pelton et al. 1991). TGF-β and its receptors have numerous functions such as regulating the immune response, cell proliferation and differentiation (Taipale Saharinen and Keski-Oja 1998; Haruyama et al. 2006). There are also important in regulating tooth development during the early stages (Chai et al. 1994; Chai et al. 1999; Pelton et al. 1991). Overexpression of TGF-β1 under the dentin sialoprotein pro-

moter in teeth has shown to result in a pitted and hypoplastic enamel in mice (Haruyama et al. 2006). The observed phenotype is due to an early and abnormal detachment of the ameloblast cells from the underlying dentin, and formation of cysts that contain hypomineralized matrices near the DEJ (Haruyama et al. 2006).

Smad3 is a member of Smad proteins, which are intracellular signaling molecules that mediate the signals from activin and TGF-β super family receptors to the nucleus (Yokozeki et al. 2003). Mice that lack the Smad 3 gene have severely hypomineralized enamel in their molars and incisors, due to defective protein removal at the maturation stage (Yokozeki et al. 2003). The defective enamel has a normal thickness and rod and interred structure, which indicate that the secretory stage of amelogenesis is not affected in these transgenic mice (Yokozeki et al. 2003). The Smad3 null mice also have a decreased inflammatory response and accelerated wound healing (Yokozeki et al. 2003). From the observed results it can be hypothesized that Smad3 is critical for enamel biomineralization, and that the activin and TGF-β signaling may be essential for this process to take place normally (Yokozeki et al. 2003).

Cell adhesion molecules such as laminins and integrins are strongly expressed by secretory ameloblasts and play a role in regulating ameloblast cytodifferentiation (Bei 2009). Laminins are a family of extra-cellular matrix proteins that play a role in cell differentiation, migration, and adhesion (Yoshiba et al. 1998). Laminin-5 (Laminin-332), in specific, regulates epithelial cells adhesion and mobility via integrins $\alpha 6\beta 4$ and $\alpha 3\beta 1$, and its isoforms are expressed in the basement membrane of secretory ameloblasts (Yoshiba et al. 1998; Ryan et al. 1999). Laminin-5 plays a role in the late stage differentiation of ameloblast cells and thus, when it is mutated, the ameloblast terminal differentiation is affected and hypoplastic enamel is formed (Ryan et al. 1999). The seceretory amloblasts of mice with a mutated laminin 5 alpha 3 produce very little enamel matrix (Ryan et al. 1999, Bei 2009). It is postulated that the laminin-5 interactions with the integrin $\beta 4$ is critical in stabilizing the ameloblast cells structure and architecture (Ryan et al. 1999, Bei 2009). The absence of laminin 5 alpha 3 chain alters the ameloblast adhesion and subsequently its structure and function, which leads to a formation of defective and reduced enamel (Ryan et al. 1999).

Transcription factors have also been shown to be critical for amelogenesis. The homeobox gene Msx2 is part of the Msx homeobox gene family and is expressed at numerous tissues including the eyes, hair follicles, and teeth (Satokata et al. 2000; Bei et al. 2004; Bei 2009). Mutations in Msx2 gene affect the development of dentition in several different ways (Satokata

et al. 2000). Mice lacking the homeobox gene Msx2 exhibit defects in cusp morphpogenesis, and have severely misshaped teeth (Bei et al. 2004). Furthermore, in the Msx2 knockout mice, the secretory ameloblasts produce a severely hypoplastic enamel (Bei et al. 2004). The ameloblast cells of these mice lose their cell-cell adhesion, and exhibit a poorly formed hemidesmosomes, indicating the critical role of Msx2 in cell adhesion (Bei et al. 2004). Moreover, Msx2 controls the expression of laminin 5 alpha 3 chain in secretory ameloblasts (Bei et al. 2004). Interestingly, the mutations in Msx2 and laminin 5 alpha 3 chain both result in reduced enamel deposition and a very similar phenotype, supporting the hypothesis that these genes function within the same genetic pathway (Bei et al. 2004; Bei 2009).

Table 1.3: Mutations in transgenic mice that cause enamel defects

Gene	Mutation	Enamel phenotype	References
Sp3	Knockout	Hypoplastic	Bouwman et al. (2000)
Sp6	Knockout	Hypoplastic	Nakamura et al. (2008), Ruspita et al. (2008)
Msx2	Knockout	Hypoplastic	Satokata et al. (2000), Bei et al. (2004)
Smoothened	K14 conditional knockout	Hypoplastic	Gritli-Linde et al. (2002)
Lama3	Knockout	Hypoplastic	Ryan et al. (1999)
TGFβ1	Overexpression (DSPP conditional knockout)	Hypoplastic	Haruyama et al. (2006)
Eda	Overexpression (under K14 promoter)	No enamel	Mustonen et al. (2004)
Wnt3	Overexpression (under K14 promoter)	No enamel	Millar et al. (2003)
Follistatin	Overexpression (under K14 promoter)	No enamel	Wang et al. (2004)
Follistatin	Knockout	Ectopic enamel	Wang et al. (2004)
Gdnf	Knockout	No enamel	de Vicente et al. (2002)
Periostin	Knockout	Enamel defect	Rios et al. (2005); Ma et al. (2011)
Ameloblastin	Knockout	No enamel	Fukumoto el al. (2005)
Smad 3	Knockout	Hypomineralized	Yokozeki et al. (2003)
Connexin 43	Dominant negative	Hypoplastic	Dobrowolski et al. (2008)

1.8 Structure and function of integrins

Integrins comprise a large family of heterodimeric cell surface protein receptors, found to be critical for embryogenesis (Campbell and Humphries 2011; Giancotti 1997; Hynes 2004). They mediate cell-to-cell or cell-to-matrix adhesion and signaling in most cell types (Campbell and Humphries 2011; Giancotti and Ruoslahti 1999; Hynes 2004). Integrins are composed of two non-covalently associated transmembrane subunits, α and β, which typically have a long extracellular domain and a short intracellular domain (Hynes 1992). The integrin $\alpha 6\beta 4$ is an exception in this regard, as it possesses a long cytoplasmic tail (Suzuki and Naitoh 1990; Tamura et al. 1990). There are 18 α and 8 β subunits in vertebrates that dimerize to form 24 different integrins, each with a different tissue distribution, binding specificity, and signalling properties (Barczyk Carracedo and Gullberg 2010; Hynes 2002; Hynes 2002).

In order for integrins to get activated, intracellular cell signals need to interact with the cytoplasmic domain of integrins leading to a conformational change in the extracellular portion of the integrin (Larjava et al. 2011). The inactive bent integrin changes into its activated extended form, which exposes the ligand binding site (Larjava et al. 2011). This process is referred to as inside-out activation (Larjava et al. 2011).

Upon activation, integrins bind to a number of different ligands, many of which are shared targets amongst the family members (Bouvard et al. 2001; Hynes 1992). In addition to binding to extracellular matrix (ECM) ligands, integrins may also serve as receptors for many viruses and bacteria (Hynes 2002).

The cytoplasmic tails of the integrins function in the binding and reorganization the actin cytoskeleton of a cell, which initiates a positive feedback system in which more integrins cluster and further organization of the actin filaments takes place. In the case of $\alpha 6\beta 4$ integrin, it binds to intermediated filaments rather than actin filaments due its large intracellular domain (Hynes 2002; Giancotti and Ruoslahti 1999; Tarone et al. 2000). This results in the formation of aggregates known as "focal adhesions", composed of extracellular matrix proteins, integrins, and intra- and extracellular components of the cytoskeleton (Burridge and Chrzanowska-Wodnicka 1996). The focal adhesions are tightly controlled and allow for cell migration and adhesion to occur.

Besides their role in organization of the cytoskeleton and their integration with the ECM, integrins play a pivotal role in transducing signals through the cell membrane, in either direction (Giancotti and Ruoslahti 1999). These

signals have been found to regulate cell survival, proliferation, and cycle (Giancotti 1997; Hynes 1992).

The critical role that integrins play in biological processes is clearly demonstrated in integrin deficient and transgenic animals. Ablation of integrin genes results in various functional deficiencies such as defects in the kidneys, lungs and skin, to embryonic inviability, to name a few examples (Bouvard et al. 2001).

Research regarding integrin expression during tooth development has shown that tooth epithelium express $\alpha 6$, αv, $\beta 1$, $\beta 4$, and $\beta 5$ integrin subunits (Salmivirta et al. 1996). Furthermore, the secretome of rat incisor enamel organ has been reported to include the $\beta 6$ integrin transcript, but not much is known about αv$\beta 6$ integrin in enamel formation (Moffatt et al. 2006).

1.9 αvβ6 integrin

αv$\beta 6$ integrin is exclusively an epithelial receptor which binds to its ligands through the arginine-glycine-aspartic acid (RGD) motif (Breuss et al. 1993; Breuss et al. 1995; Thomas Nystrom and Marshall 2006). αv$\beta 6$ integrin's RGD containing ligands include fibronectin (Busk Pytela and Sheppard 1992), vitronectin (Huang et al. 1998), tenascin (Prieto Edelman and Crossin 1993), and fibrillin-1 (Jovanovic et al. 2007). The integrin also binds to RGD on latency associated peptides (LAP) of transforming growth factor- $\beta 1$ (TGF-$\beta 1$) (Munger et al. 1999) and TGF-$\beta 3$ (Annes Rifkin and Munger 2002), viral capsids of foot-and-mouth disease virus (FMDV) (Miller et al. 2001), coxsackievirus A9 (CAV9) (Williams et al. 2004), and human parechovirus 1 (HPEV 1) (Seitsonen et al. 2010).

While the αv subunit can pair with multiple β subunits, the $\beta 6$ subunit binds exclusively with the αv unit to form the αv$\beta 6$ integrin (Hynes 2002). The $\beta 6$ integrin has a 788 amino acid sequence, and is distinct from other related beta subunits due to its unique eleven amino acid carboxyl-terminal extension (Sheppard et al. 1990).

The αv$\beta 6$ integrin is normally expressed at low levels in adult tissues, but it is upregulated during development, wound healing, and inflammation, (Breuss et al. 1995; Clark et al. 1996; Haapasalmi et al. 1996; Hahm et al. 2007; Hakkinen et al. 2000; Larjava et al. 1993) as well as in more severe pathologies such as chronic skin wounds (Hakkinen et al. 2004) and in a variety of cancers (Hamidi et al 2000; Thomas Nystrom and Marshall 2006). It is also constitutively expressed in junctional epithelium of teeth (Ghannad et al. 2008; Larjava et al. 2011), and the hair follicles of normal

adult tissue (Xie et al. 2009).

Activation of TGF-β1 is considered to be one of the main functions of αvβ6 integrin *in vivo*. TGF-β1 has a role in immunoregulation, where it can act as either a pro-inflammatory cytokine, or an anti-inflammatory cytokine, depending on the cell type (Li Sanjabi and Flavell 2006; Wahl et al. 2004). Mice deficient in the β6 integrin develop mild inflammation in their skin and lungs, due to increased infiltration of macrophages and lymphocytes (Huang et al. 1996). Furthermore, the macrophages of these mice are deficient in clearing the phospholipids in their lungs (Koth et al. 2007). In addition, β6 knockout mice develop periodontal disease, in which chronic inflammation, pocket formation, and bone loss are observed (Ghannad et al. 2008; Larjava et al. 2011). These results are attributed to reduced αvβ6 mediated TGF-β1 activation, and suggest that αvβ6 integrin functions to modulate inflammatory response in the epithelial cells (Ghannad et al. 2008; Huang et al. 1996; Koth et al. 2007; Larjava et al. 2011). Moreover, β6 knockout mice show protection from pulmonary fibrosis (Munger et al. 1999; Puthawala et al. 2008), renal fibrosis (Hahm et al. 2007; Ma et al. 2003), liver fibrosis (Patsenker et al. 2008; Wang et al. 2007), and TGF-β-mediated pulmonary edema (Pittet et al. 2001), which is due to reduced TGF-β activity.

1.10 TGF-β activation

TGF-β is a multifunctional cytokine that regulates cell growth, inflammation, immune function, matrix synthesis, and apoptosis (Taipale Saharinen and Keski-Oja 1998). It functions by binding to its receptor and activating an intracellular signalling cascade that results in alteration of gene expression. There are three isoforms of TGF-β (TGF-β1, -2, and -3), which are all secreted as inactive complexes. As such, TGF-β first needs to be activated in order to be able to bind to its receptor and exert its effect. The αvβ6 integrin binds and activates latent TGF-β1 and TGF-β3 (Annes Rifkin and Munger 2002; Annes et al. 2004).

TGF-β is synthesized as a homodimeric pro-TGF-β that is covalently linked to another propeptide termed latency associated peptide (LAP) (Massague Blain and Lo 2000; Worthington Klementowicz and Travis 2011). Even though LAP is cleaved from mature TGF-β in the Golgi, the TGF-β remains non-covalently attached to LAP upon its secretion (Dubois et al. 1995).This associated LAP prevents signalling by blocking the TGF-β receptor, and together with TGF-β it forms the small latent complex (SLC) (Lawrence et al. 1984). In most of the cases however, TGF-β is secreted as

part of the large latent complex (LLC) (Dallas et al. 1994; Miyazono et al. 1991; Taipale Saharinen and Keski-Oja 1998). LLC is formed when the SLC (LAP- TGFβ) is covalently associated with latent TGF-β-binding protein (LTBP) (Saharinen and Keski-Oja 2000). LTBP binds to proteins in ECM, and therefore anchors the latent TGF-β to the ECM (Hyytiainen Penttinen and Keski-Oja 2004). The LAP needs to be dissociated from TGF-β in order for TGF-β to get activated. This dissociation is a highly regulated process, and is termed latent TGF-β activation (Annes et al. 2004). Although a number of different processes such as proteases that degrade LAP (Kojima Nara and Rifkin 1993; Yu and Stamenkovic 2000), thrombospondin-1 (Crawford et al. 1998), and reactive oxygen species (Jobling et al. 2006) can activate latent TGF-β, integrins have been recognized as the key activators (Aluwihare et al. 2009; Nishimura 2009; Worthington Klementowicz and Travis 2011; Yang et al. 2007). The αvβ6 mediated TGF-β1 activation is a protease independent mechanism during which an induction of a conformational change in the LAP occurs (Annes et al 2004).

αvβ6 integrin recognizes and binds to the RGD motif in the LAP of the latent complex (Annes et al. 2004). This leads to a conformational change of the LLC and allows activated TGF-β1 to access its receptors (Annes et al. 2004; Fontana et al. 2005; Wipff et al. 2007; Wipff and Hinz 2008; Worthington Klementowicz and Travis 2011). Activation of TGF-β1 through this mechanism is dependent on integrin ability to bind to the actin cytoskeleton of the cell, which generates a retractile force and results in a conformational change in LLC. TGF-βs bind to type I and type II receptors (TGF-βRI and TGF-βRII), which in turn phosphorylate (activate) Smad2 and Smad3 (Moustakas and Heldin 2008). Phosphorylated Smad2 and Smad3 then form a complex with Smad4, which translocates to the nucleus to regulate gene transcription (Kang Liu and Derynck 2009). In addition to the Smad signaling pathway, TGF-β receptors can signal via alternative pathways such as mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3K), and Rho-like GTPase pathway (Zhang 2009). TGF-β1 knockout animals die a few weeks after birth from multifocal inflammatory disease, due to massive infiltrations of macrophages and lymphocytes in different organs (Shull et al. 1992).

Although no enamel defects have been reported with TGF-β1 knockout animals, mice that lack Smad3 gene have phenotypes that mimic hypomineralized amelogenesis imperfecta (Yokozeki et al. 2003).

Chapter 2

The role of αvβ6 integrin in enamel biomineralization

2.1 Introduction

Enamel is the hardest mineralized tissue in the body and the only calcified tissue that is produced by epithelium-derived cells, namely ameloblasts. Amelogenesis consists of secretory, transition and maturation stages (Simmer et al. 2010). During the secretory stage, ameloblasts secrete enamel proteins such as amelogenin (the most abundant enamel matrix protein; Eastoe, 1979), ameloblastin (Krebsbach et al. 1996) and enamelin (Hu et al. 1997) into the enamel matrix. This extracellular matrix undergoes enzymatic modification by enamelysin (MMP20) and kallikrein 4 (KLK4) in the transition and maturation stages, which results in the formation of a mature enamel that is mainly composed of hydroxyapatite crystallites and a minor amount of residual proteins (Bartlett et al., 1996; Nanci and Smith, 2000). Mutations in amelogenin, enamelin, MMP20 and KLK4 genes all cause human hereditary amelogenesis imperfecta (AI), in which both enamel formation and its mineralization are affected (Hu et al. 2007). This leads to extensive wear and decay in both the primary and permanent dentition, which may result in tooth loss at a young age or require extensive restorative procedures to prevent further decay and/or attrition (Crawford et al. 2007).

During amelogenesis, the apical ameloblast plasma membrane directly abuts against the matrix and the developing enamel crystals (Nanci and Smith, 2000). Although integrins mediate cell-matrix adhesion and signaling in most cell types (Hynes 2004), the receptors of ameloblasts that mediate ameloblast-matrix adhesion, matrix organization, and signaling are not well characterized. The rat incisor enamel organ has been reported to express the β6 integrin transcript, but its role in enamel formation is not known (Moffatt et al. 2006). Integrin αvβ6 is an epithelial cell-specific integrin that binds to the arginine-glycine-aspartic acid (RGD) amino acid motif in its ligands (Breuss et al. 1993), which include fibronectin, tenascin-C, vit-

ronectin and the latency-associated peptide (LAP) of transforming growth factor-β1 (TGF-β1) and TGF-β3. Its binding to the LAP of latent TGF-β1 activates the cytokine (Munger et al. 1999). This integrin-mediated activation plays an important anti-inflammatory role in vivo (Yang et al. 2007), as it regulates experimental TGF-β1-dependent fibrosis in various organs (Hahm et al. 2007; Horan et al. 2008; Patsenker et al. 2008). Furthermore, αvβ6 integrin is expressed in junctional epithelial cells that mediate gingival soft tissue adhesion to tooth enamel (Ghannad et al. 2008). Interestingly, loss of β6 integrin in mice is associated with characteristics of human periodontal disease, suggesting that αvβ6 integrin plays a role in protecting periodontal tissues from inflammatory changes leading to periodontal disease (Ghannad et al. 2008). Junctional epithelium is derived from cells arising from the reduced enamel epithelium during tooth eruption (Schroeder and Listgarten 1977). In the present study, therefore, we hypothesized that αvβ6 integrin is also expressed in ameloblasts and that lack of its expression is associated with enamel defects. We show that mice deficient in αvβ6 integrin have hypomineralized enamel and show excessive accumulation of amelogenin in the mineralizing enamel matrix.

2.2 Results

2.2.1 Teeth of *Itgb6-/-* mice have severe attrition and abnormal enamel surface

Compared to wild-type (WT) mice, *Itgb6-/-* mice had no obvious differences in tooth development or eruption, and there was no malocclusion. However, when incisors of *Itgb6-/-* mice were examined more closely, it became obvious that they differed from those of WT mice. The maxillary incisors of WT mice had a smooth and yellowish surface, whereas *Itgb6-/-* mouse maxillary incisors lacked the yellow pigmentation and were abnormally white. In addition, mandibular incisors of *Itgb6-/-* mice appeared chalky and the tips were noticeably more rounded than those of their WT counterparts (Figure 2.1A,B).

Examination of defleshed mandibles from 12-month-old mice using a dissecting microscope or after taking high-resolution radiographs showed that whereas WT mouse molars had prominent and pointed cusps on all three molars (Figure 2.1C,E,G), the molar cusps of the *Itgb6-/-* mice had extensive wear at their occlusal surfaces (Figure 2.1D,F,H) similar to their blunted incisors. This severe attrition of molar cusps was highly evident in the scanning electron micrographs, displaying flattened and heavily worn *Itgb6-/-*

mouse molar cusps (Figure 2.1H). While the WT enamel was smooth, the enamel of *Itgb6-/-* mice showed extensive pit formation and roughness on the sides of the molars (Figure 2.1I,J). The rate and amount of attrition in the molar teeth was further investigated in different age groups. As expected, as the mice aged the attrition rate increased in both the WT and *Itgb6-/-* mice; however, the rate of attrition was much faster in the *Itgb6-/-* group (Figure 2.1K). The degree of attrition in the *Itgb6-/-* mice reached the maximum level already by 6 months when the cusps were almost completely worn down (Figure 2.1K).

Next, we investigated whether the enamel phenotype in the *Itgb6-/-* mice could be rescued by re-expressing β6 integrin. For this purpose, the *Itgb6-/-* mice were bred with mice over-expressing human β6 integrin under the cytokeratin 14 (K14) promoter in addition to their own endogenous mouse β6 integrin (Häkkinen et al. 2004). Offspring carrying only human β6 integrin on the knockout background were selected to represent the β6 integrin rescue mice (Figure 2.1L; integrin expression in the ameloblast layer of these animals is presented in Figure 2.6A) The incisors of the 'over-expressing' and the 'rescue' mice appeared macroscopically normal (data not shown). The average attrition rate of 6-month-old *Itgb6-/-* mice was significantly higher when compared to the 'rescue' and 'over-expressing' mice of the same age, indicating that the enamel attrition is effectively rescued by human β6 integrin gene in the *Itgb6-/-* mice (Figure 2.1M). Moreover, over-expression of β6 integrin did not seem to affect enamel formation.

2.2.2 Enamel prism structure and mineralization are severely affected in *Itgb6-/-*

To determine the specific nature of the enamel defects in *Itgb6-/-* mice, we first observed enamel microstructure. Electron micrographs of surface acid-etched incisors demonstrated a significant difference in prism structure and organization between the WT and *Itgb6-/-* enamel (Figure 2.2A,B). In WT mouse incisors, a parallel and well-organized enamel prism structure in alternating rows was present (Figure 2.2A). Interestingly, in the *Itgb6-/-* incisors, the enamel prism organization was completely lost; the enamel was somewhat layered but showed no regular repeating pattern of prism structure (Figure 2.2B).

Next, we compared enamel mineral density in WT and *Itgb6-/-* mice. Micro-CT images were taken from 14-day-old *Itgb6-/-* and WT mouse mandibular molars. While the mutant mice teeth appeared generally anatomically normal, the X-ray density of the enamel was greatly reduced in both the 1st

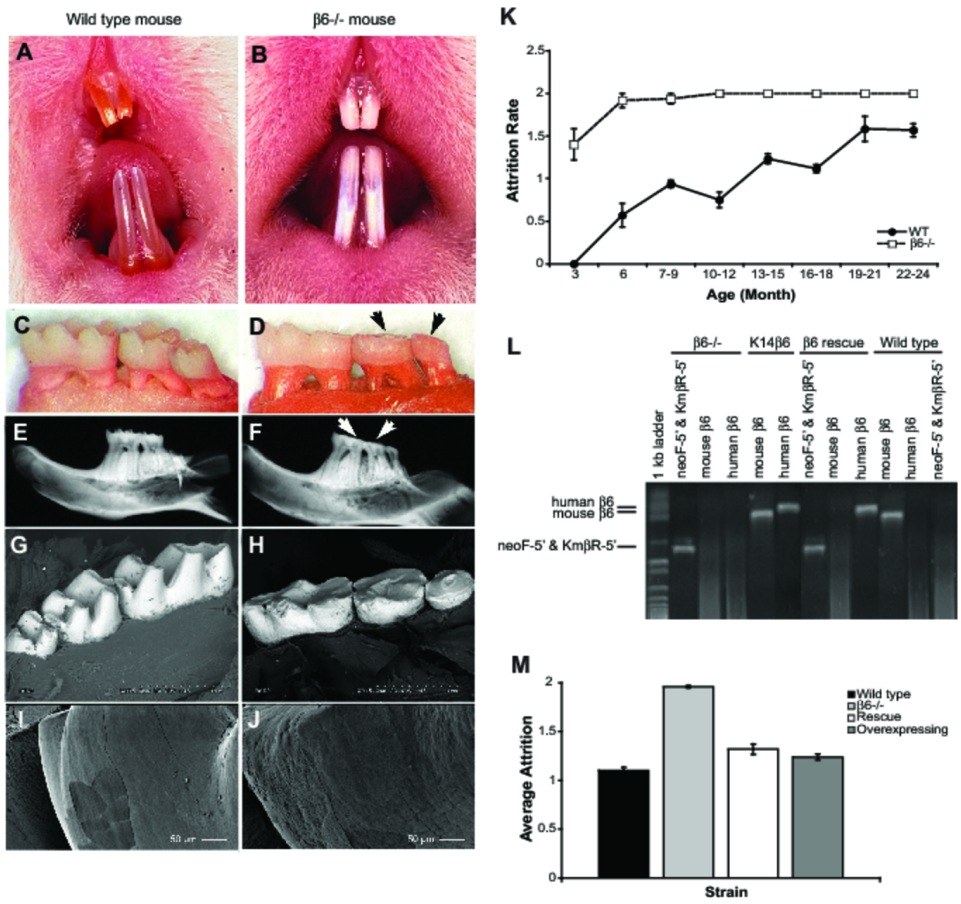

Figure 2.1: **Teeth from 6-month-old *Itgb6-/-* mice show severe attrition**. (A, B) Incisors of the *Itgb6-/-* (β6-/-) mice show considerable wear at their tips, a chalky appearance and an absence of yellow pigmentation as compared to WT incisors. (C, D) Severe attrition is also observed in the *Itgb6-/-* mandibular molars. (E-H) High-definition radiographs and backscattered electron SEM images of defleshed mandibles demonstrate occlusal wear to the level of dentin in the *Itgb6-/-* molars. (I, J) In secondary electron SEM images, lateral enamel surfaces in *Itgb6-/-* mouse molars appear rough and pitted as compared to those in WT mice. (K) In WT mice, the attrition rate increases with age reaching a score (see Methods) of one in about twelve months. However, attrition in *Itgb6-/-* (β6-/-) mice reaches the maximum score of two in six months. (L) PCR genotyping shows that WT mice express the murine β6 integrin while there is no expression in *Itgb6-/-* animals (β6-/-). K14β6 mice demonstrate the presence of both human and murine β6 integrin. Integrin β6-deficient mice were cross-bred with K14β6 mice to create a β6 integrin rescue line (β6 rescue) carrying only the human β6 integrin gene. The knockout construct was identified by primers for neoF-5' and KmβR-5'. (M) Significant clinical attrition observed in the *Itgb6-/-* (β6/-) mice compared to WT mice can be rescued by human β6 integrin. WT, N= 167; β6-/-, N=124; rescue, N=60; overexpressing, N=99.

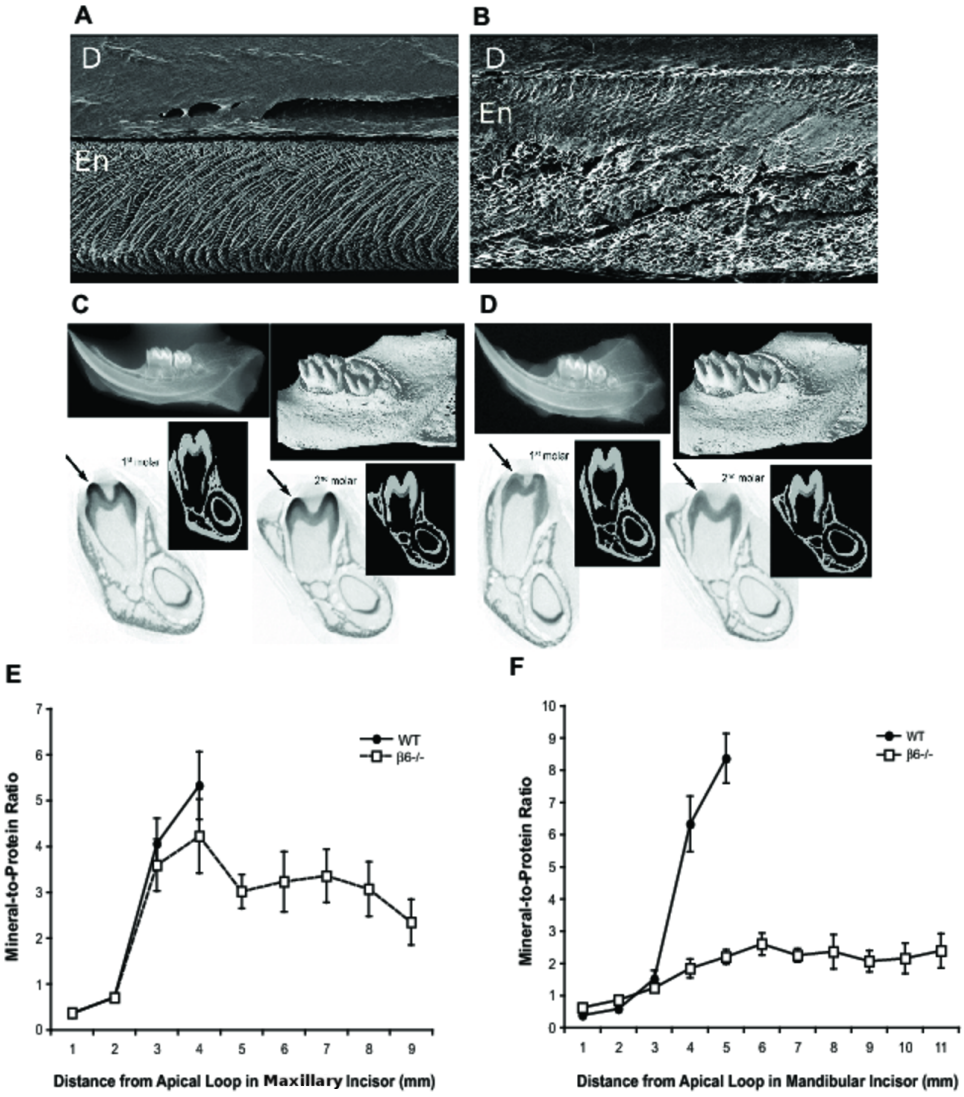

Figure 2.2: **Enamel prism structure and mineralization are severely affected in *Itgb6-/-***. (A) Enamel in WT mice shows a well-organized and regular prism structure. (B) In *Itgb6-/-*(*β*6-/-) enamel, the prism structure is completely lost and irregular. D, dentin, En, enamel. Micro-CT images of mandibular specimens from 14-day-old WT (C) and *Itgb6-/-* (D) mice show that the general development, relationship with alveolar bone, and eruption of both molars and the incisor appear normal in *Itgb6-/-* mice. In longitudinal sections of both the 1st and 2nd molars, the mineral density of enamel appears greatly reduced (less black/dark grey) in *Itgb6-/-* mice (arrows). Mineral-to-protein ratios in the maxillary (E) and mandibular (F) incisors of 7-week-old WT and *Itgb6-/-*(*β*6-/-) mice (n=10) derived from 1-mm strips of developing enamel cut from the apical loop of the incisors and processed for protein and mineral measurements. Strips 1-2 represent secretory stage enamel, strips 3-4 maturation stage enamel and strips 5-11 mature hard enamel.

and 2nd molars of the *Itgb6-/-* mice compared to the WT (Figure 2.2C,D). To quantify the difference in mineralization during various stages of enamel formation, we measured the mineral-to-protein ratio of the maxillary and mandibular incisors in both mouse lines by an ashing method. Near the apical loop of the incisors, corresponding to the secretory stage, the mineral-to-protein ratio was similar in WT and *Itgb6-/-* mice (Figure 2.2E,F). However, as the distance from the apical loop increased (maturation stage starts about 3 mm from the apex), the mineral-to-protein ratio of *Itgb6-/-* mouse incisors became drastically decreased compared to that of the WT mice. In fact, from the regions that corresponded to the maturation stage and onwards, the WT incisors were so highly mineralized that it was not possible to cut away incisor enamel strips for sampling, whereas the *Itgb6-/-* incisor enamel strips were easily removed along the entire length of the incisors (strip 9 on maxilla and strip 11 on the mandible). In additional mineral analyses using energy-dispersive X-ray spectroscopy, we studied a number of molar teeth (areas of no wear on the buccal surface) where there was no statistical difference in Ca/P ratios between *Itgb6-/-* and WT molars with the ratios obtained being typical for hydroxyapatite (data not shown).

2.2.3 Integrin β6 mRNA and protein are expressed by ameloblasts in mouse and human teeth

To demonstrate that the β6 integrin mRNA and protein are expressed in mouse teeth, in situ hybridization and immunohistochemical studies were performed. In situ hybridization of β6 integrin mRNA with the anti-sense and sense probes was performed in the secretory stage of an upper incisor from a 10-day-old WT mouse. The anti-sense probe demonstrated a strong specific signal that was localized only to the cytoplasm of ameloblasts (Figure 2.3A), whereas no signal was detected with the control sense probe (Figure 2.3B).

Next, we performed immunostaining of β6 integrin protein in frozen sections from the secretory stage of an upper incisor as well as from developing upper molar cusps from 3-day-old WT mice. In both the incisor and molar teeth, prominent β6 integrin immunoreactivity was localized to the ameloblast layer (Figure 2.3C,D). Hematoxylin- and eosin-stained sections confirmed that the β6 integrin immunoreactivity was indeed localized to the ameloblast cell layer (Figure 2.3E,F).

In order to investigate whether expression of β6 integrin in ameloblasts is similar in humans, we immunostained maturation-stage ameloblasts from surgically removed, unerupted human wisdom teeth. Ameloblasts were iden-

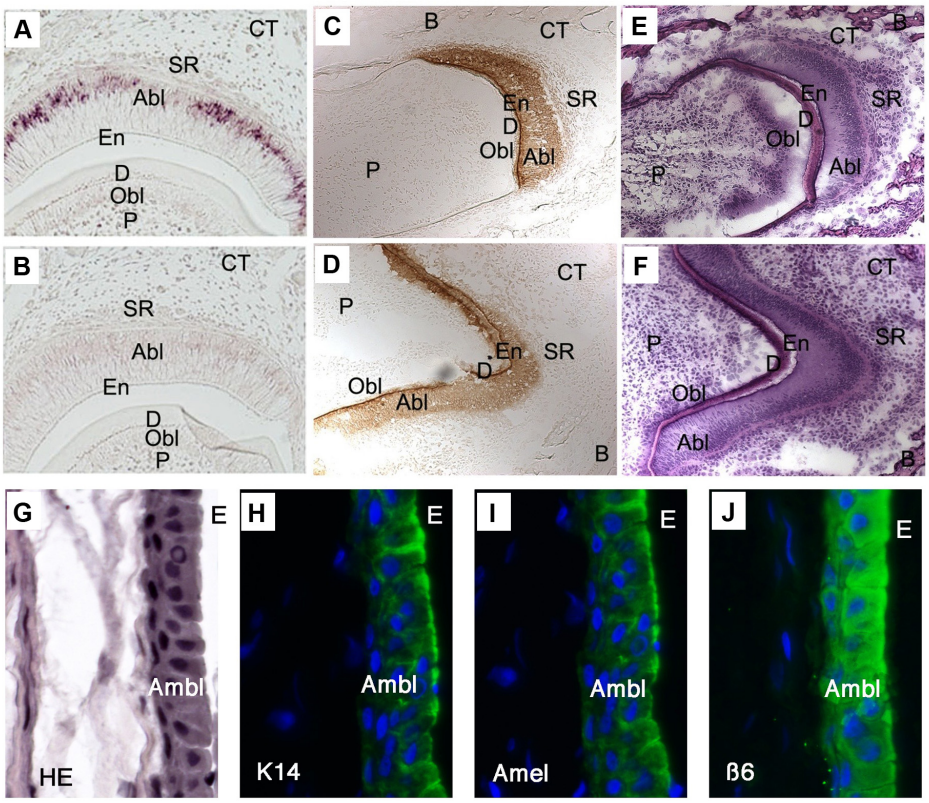

Figure 2.3: **Integrinβ6 mRNA and protein are expressed by ameloblasts in developing mouse and human teeth.** In situ hybridization of β6 integrin with anti-sense (A) and sense (B) probes in the secretory stage of a maxillary incisor from a 10-day-old WT mouse. Localization of β6 integrin protein by immunostaining in undecalcified frozen sections from the secretory stage maxillary incisor (C) and a developing maxillary molar cusp (D) from a 3-day-old WT mouse. (E, F) Parallel sections to C and D, respectively, stained with H&E. (G) Ameloblasts are present on extracted developing human third molars (HE staining), and they show positive staining for K14 (H), amelogenin (I) and β6 integrin (J) by immunohistochemistry. Abl/Ambl, ameloblast layer; P, pulp tissue; Obl, odontoblast layer; En/E, forming enamel; D, forming dentin; SR, stellate reticulum; CT, connective tissue; B, bone.

tified in decalcified sections by morphology as well as by a positive signal for K14 and amelogenin (Figure 2.3G-I). The ameloblast layer was strongly labeled with the β6 integrin antibody (Figure 2.3J), indicating that expression of αvβ6 integrin is conserved in human ameloblasts.

2.2.4 Expression of amelogenin and enamelin is significantly increased in the *Itgb6-/-* ameloblast layer

In order to identify the gene expression profile associated with the disturbed mineralization of *Itgb6-/-* enamel, we removed incisor enamel organs of adult *Itgb6-/-* and WT mice from both the secretory and maturation stages. A heat map of the differentially expressed genes was generated (Figure 2.4A). These data demonstrated significant differences in gene expression profiles in enamel organs between the WT and *Itgb6-/-* mice. Using the GOrilla Gene Ontology analysis tool, two of the biological processes that show significant differences were identified as 'regulation of biomineralization' and 'osteoblast differentiation' (Figure 2.4B). The microarray gene expression results for 'cellular components' and 'biological processes' were further analyzed using DAVID (Database for Annotation, Visualization And Integrated Discovery). In both categories, those genes with significant differences in their expression, including genes relevant to biomineralization, were graphed in a pie chart (Figure 2.4C,D, respectively). The rest of the genes were grouped in the 'other' category. For the 'cellular component' category, differentially regulated genes included genes associated with extracellular matrix or cell surface. For the 'biological process' category, gene expression for defence, immune response, wounding, cell-cell signaling, endocytosis, tissue morphogenesis and biomineralization differed significantly between the WT and *Itgb6-/-* mice enamel organs.

The gene profiling analysis showed that 67 genes were down-regulated and 171 genes up-regulated in *Itgb6-/-* enamel organs compared to WT enamel organs (absolute fold change >1.6; data not shown). Interestingly, the two most up-regulated genes in *Itgb6-/-* enamel organs were amelogenin (*Amelx*; 21-fold) and enamelin (*Enam*; 7.6-fold; Figure 2.5A). Other highly upregulated genes were progressive ankylosis gene (*Ank*; 5-fold) and RNA motif binding proteins (RBMs; data not shown). Expression of *Klk4* was also increased, whereas ameloblastin (*Ambn)*, MMP20 (*Mmp20*) and amelotin (*Amtn*) remained unchanged (Figure 2.5A and data not shown). The expression of β1 and β4 integrin genes was not changed, suggesting that there was no compensatory change in other integrins in the absence of β6 integrin (Figure 2.5A). Also, the level of ameloblast marker gene K14 was unchanged.

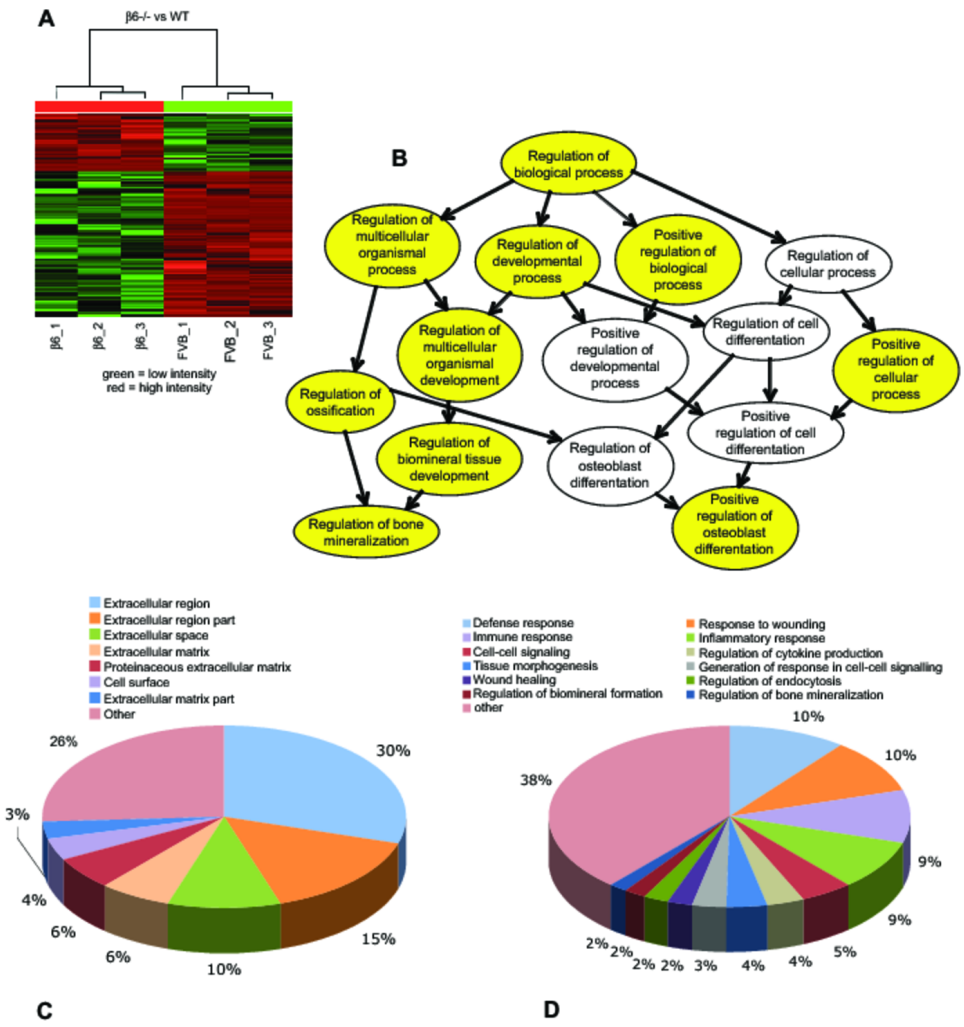

Figure 2.4: **Gene expression profiling of enamel organs from 6-month-old WT and *Itgb6-/-* mice.** (A) Heat map of the differentially expressed genes from pooled enamel organs (the groups consisted 4-6 mice each and were designated as FVB_1-3 for WT and β6_1-3 for the *Itgb6-/-* mice). (B) Differentially regulated biological processes in enamel organs between WT and *Itgb6-/-* mice analyzed using the GOrilla gene ontology analysis tool ($p>10^{-3}$ for the white circles and $p=10^{-3}$-10^{-5} for the yellow circles); Differentially regulated cellular components (C) and processes (D) between WT and *Itgb6-/-* mice analyzed by the DAVID system.

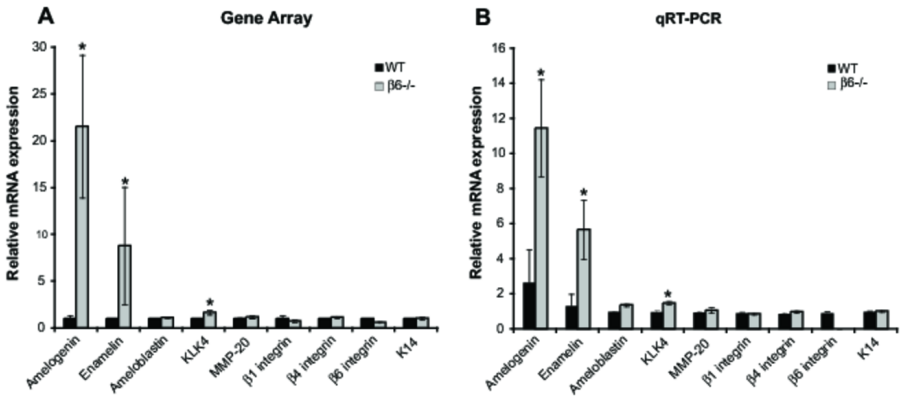

Figure 2.5: **Relative gene expression of selected enamel genes in 6-month-old WT and*Itgb6-/-* mice based on gene profiling (A) and confirmation with real-time RT-PCR (B).** Expression of amelogenin, enamelin and KLK4 genes are significantly upregulated in *Itgb6-/-*(*β*6-/-) enamel organs compared to WT enamel organs. Expression of ameloblastin, MMP20, *β*1 or *β*4 integrin and ameloblast marker K14 genes are not significantly altered. Truncated *β*6 integrin mRNA expressed in the *Itgb6-/-* organs was recognized by the gene array probes (A) but was not amplified by the PCR primers specifically designated to detect the translated mRNA (B).

Among the genes that showed the strongest down-regulation in the *Itgb6-/-* enamel organ, were arrestin domain containing 3 protein (*Arrdc3;* 8fold), photocadherin-21 (*Pcdh21*; -5.9-fold) and *Fam20B* (family with sequence homology 20, member B; -2.9-fold; data not shown). Regulation of these genes in the *Itgb6-/-* enamel organ may contribute to the hypomineralized enamel phenotype in these animals but their role needs to be further investigated.

Next, we verified the expression levels of amelogenin, enamelin and other key molecules associated with enamel mineralization using quantitative RT-PCR (Figure 2.5B). Among the tested mRNAs, only the expression of *Amelx*, *Enam* and *Klk4* showed significant increases both in gene arrays and in RT-PCR (Figure 2.5A,B).

We then verified the integrin expression profiles of the incisor enamel organs of 3-6-month-old WT, *Itgb6-/-* and *β*6 integrin rescue mice by Western blotting. As expected, *Itgb6-/-* incisors were negative for *β*6 integrin, whereas it was expressed in the WT animals (Figure 2.6A). In 'rescue' and

'overexpressing' mice, β6 integrin expression was elevated due to the constitutive K14 promoter activity in the enamel organs. Confirming the qRT-PCR results, the expression of β1 integrin was similar in all mouse lines. However, the amount of αv integrin protein, which partners with β6 integrin and others, was reduced in the *Itgb6-/-* enamel (-2.5-fold; Figure 2.6A) likely because of intracellular degradation due to lack of receptor dimerization.

2.2.5 Accumulation of amelogenin protein in the ameloblast layer and enamel of *Itgb6-/-* mice

Analysis of protein expression in incisor enamel organs of 3-6-month-old WT and *Itgb6-/-* mice by Western blotting revealed strong abnormal accumulation of amelogenin in the incisors of *Itgb6-/-* mice (on average 10-fold; Figure 2.6A,C). The main amelogenin bands were between 20-25 kDa, which is consistent with major cleaved amelogenins. Consistent with the molar attrition data, the amelogenin accumulation in the incisors of the *Itgb6-/-* mice was rescued by human β6 integrin expression (Figure 2.6A), showing that the accumulation of amelogenin was specifically attributable to β6 integrin deficiency. The expression of MMP20 and KLK4 was also slightly increased in the *Itgb6-/-* mice (Figure 2.6B,D). The protein levels of enamelin were not tested because of the lack of availability of a specific antibody for mouse enamelin.

Because one of the main functions of αvβ6 integrin is to activate TGF-β1, we compared the phosphorylation and expression levels of Smad3, a downstream target of TGF-β1, in enamel organs. While TGF-β1 effectively induced pSmad3 phosphorylation in cultured ameloblast-like cells (not shown), there was no significant difference in the expression or phosphorylation of Smad3 between the WT and *Itgb6-/-* enamel organs (Figure 2.6B), suggesting that the mineralization defect and accumulation of amelogenin in the *Itgb6-/-* enamel is not attributable to impaired TGF-β1 signaling.

To visualize the organization of the ameloblast layer, undecalcified histological sections from the late secretory stage and early maturation stage incisors of 14-day-old *Itgb6-/-* and WT mice were compared. In WT mice, the ameloblast layer was well organized against mineralizing enamel (Figure 2.7A,C). In the *Itgb6-/-* mice, the ameloblast layer was similarly organized but showed abnormal accumulation of amorphous unmineralized matrix between the cells as well as between the ameloblasts and the forming enamel surface (Figure 2.7B,D). In order to identify this material, we performed transmission electron microscopy (TEM) and immunogold la-

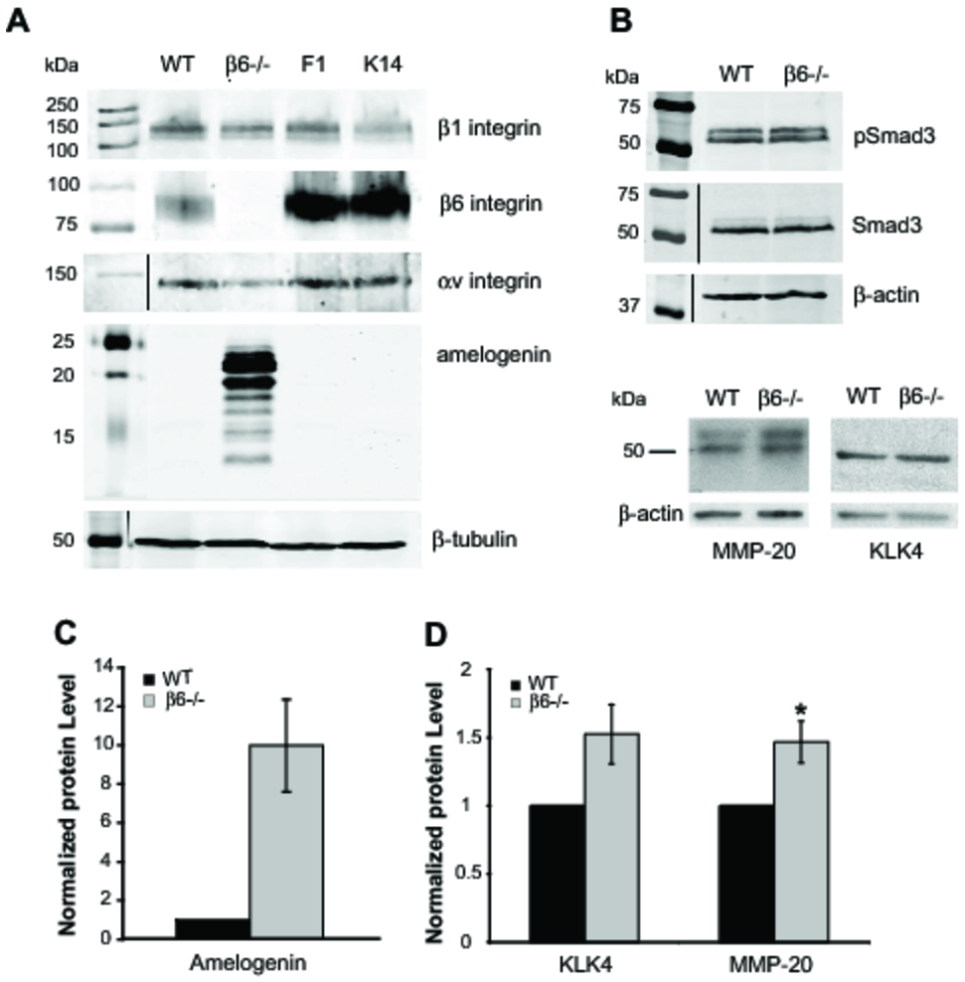

Figure 2.6: **Amelogenin protein is overexpressed in *Itgb6-/-* enamel organs.** (A) Western blots of β1, β6 and αv integrin as well as amelogenin in WT, *Itgb6-/-* (**(**β6-/-), β6 rescue (F1) and β6 overexpressing (K14) enamel organs of 3-6-month-old mice. (B) Expression and phosphorylation of pSmad3 and MMP20 and KLK4 expression in WT, *Itgb6-/-* (β6-/-) enamel organs. (C) Normalized amelogenin expression relative to β-actin. (D) Quantification of KLK4 and MMP20 Western blots relative to β-actin. (C, D) n = 3-4 pooled samples representing 4-6 mice each; mean + s.d. is shown.

beling for amelogenin localization. Ameloblasts in the WT mouse showed typical organization and secretion of enamel matrix (Figure 2.7E) that subsequently became highly mineralized in the maturation stage (Figure 2.7I). In the *Itgb6-/-* ameloblast layer, amorphous protein pools appeared in cyst-like structures between ameloblasts and towards the enamel surface (Figure 2.7F,G). By immunolabeling, this material was shown to contain abundant amelogenin (Figure 2.7H). Abnormal accumulation of amelogenin was observed both between the cells and within the cells. Electron micrographs of the surface of WT mouse molar enamel revealed a smooth, well organized, and parallel crystal structure at the enamel-ameloblast border (Figure 2.7I) in the early maturation stage, whereas corresponding regions in the *Itgb6-/-* mouse showed an enamel surface that was irregular and layered (Fig 2.7J).

2.2.6 Integrin αvβ6 participates indirectly in the adhesion of ameloblast-like cells on amelogenin-rich matrix but not in amelogenin endocytosis

To determine whether ameloblasts use αvβ6 integrin as a cell adhesion receptor for the enamel matrix, we seeded WT mouse ameloblasts in wells coated with a porcine enamel matrix extract (EMD; over 90% amelogenin). Ameloblasts spread on EMD only in the absence of the protein synthesis blocker cycloheximide (Figure 2.8A), suggesting that their adhesion on EMD was dependent on endogenous protein synthesis. Stimulating the cells with 200 nM 12O-tetradecanoylphorbol-13-acetate (TPA), an integrin activator, further increased their adhesion and spreading by about 50% (Figure 2.8A). To determine whether this adhesion was mediated by αvβ6 integrin, cell spreading was analyzed in the presence of function-blocking anti-αvβ6 integrin antibodies. Three different antibodies blocked 35-50% of the cell spreading in a statistically significant manner (Figure 2.8B), indicating that ameloblasts deposit endogenous, amelogenin-binding extracellular matrix proteins, some of which are αvβ6 integrin ligands. As expected, these antibodies did not affect the spreading of *Itgb6-/-* ameloblasts (data not shown).

We next tested whether αvβ6 integrin is involved in the regulation of amelogenin endocytosis in mouse ameloblasts. Amelogenin endocytosis occurred in a similar fashion in both WT and *Itgb6-/-* ameloblasts (Figure 2.8C-H), suggesting that αvβ6 integrin does not play a significant role in this internalization process.

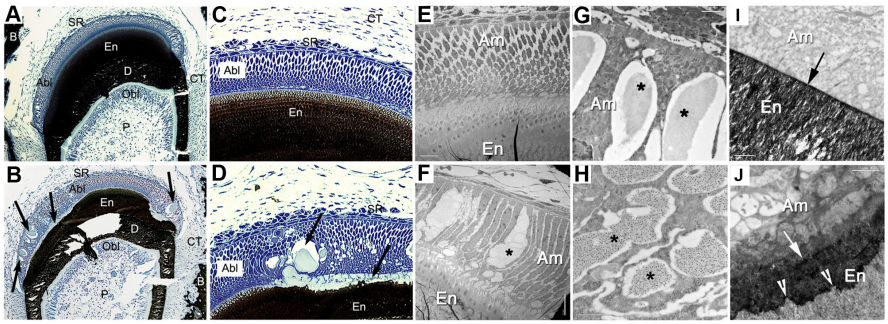

Figure 2.7: **Accumulation of amorphous matrix material between the ameloblast layer and the forming enamel, and between ameloblasts, in *Itgb6-/-* mice.** Undecalcified tissue sections from incisors of 14-day-old WT (A, C) and *Itgb6-/-* mice (B, D) stained with von Kossa reagent for mineral followed by counterstaining with toluidine blue. Compared to WT incisors, *Itgb6-/-* mice show accumulation of unmineralized extracellular matrix (arrows) between the ameloblast layer and the forming enamel surface, and between individual ameloblasts. En, enamel; Abl, ameloblast layer; D, dentin; Obl, odontoblast layer; SR, stellate reticulum; B, bone; CT, connective tissue; P, pulp tissue. Transmission electron micrographs of enamel organ of WT (E, I) and *Itgb6-/-* mice (F-H, J) showing secretory ameloblasts and enamel at the level of the second molar. Ameloblasts (Am) of the WT mouse (E) show typical organization and secretion of the enamel matrix (En) that subsequently becomes mineralized. In the *Itgb6-/-* ameloblast layer (F), amorphous protein pools appear in cyst-like structures between ameloblasts (asterisks) and near the enamel surface. Immunogold labeling for amelogenin of *Itgb6-/-* enamel organ shows abnormal amelogenin accumulation both between the cells and inside the cells (asterisks in G and H). The enamel (En) ameloblast (Am) interface in the WT maturation stage molar tooth is smooth and well mineralized and shows organized, parallel crystal structure at this boundary (I, arrow). In a corresponding molar crown region from an *Itgb6-/-* mouse, the enamel surface is less organized and irregularly mineralized with a layered appearance (J, arrow and arrowheads).

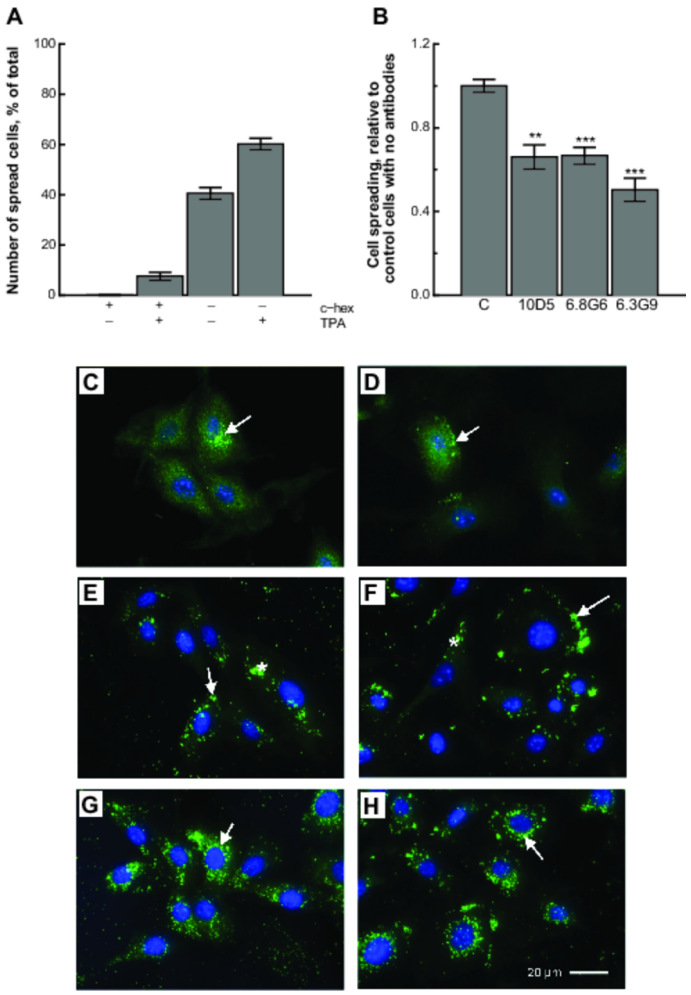

Figure 2.8: **Spreading, but not endocytosis, of ameloblast-like cells is regulated by αvβ6 integrin on amelogenin-rich enamel matrix.** (A) Ameloblast cell spreading on plates coated with 10 mg/ml of EMD in the presence or absence of 50 μM cycloheximide and 200 nM TPA. (B) WT ameloblasts were allowed to spread on EMD in the presence 200 nM TPA and three different anti-αvβ6 integrin antibodies (10D5, 50 μg/ml; 6.8G6, 10 μg/ml; 6.3G9, 10 μg/ml) and in the absence of cycloheximide. Control cells (C) were left untreated. Combined results of four experiments (mean $\pm$ s.e.m.) are shown. **, $p<0.01$; ***, $p<0.001$. Amelogenin endocytosis by WT (C, E, G) and *Itgb6-/-* ameloblasts (D, F, H). Both WT (C) and *Itgb6-/-* ameloblasts (D) show a very low level of endogenous amelogenin expression by immunostaining of the protein (green, arrows). Cell nuclei were counterstained with DAPI (blue). When incubated with 100 μg/ml EMD for 24 h, accumulation of amelogenin was distinct within the cells (E, F). The accumulation was seen in clusters on the cell edges (arrows) and in cytoplasmic regions (asterisk) for both cell types. In the presence of both EMD and 20 μM E64d (G, H), the staining of amelogenin became more punctate and diffuse (arrow). There was no difference in the pattern of endocytosis between *Itgb6-/-* and WT cell lines, indicating that β6 deficiency does not affect amelogenin endocytosis.

2.3 Discussion

Epithelial-cell integrins regulate a variety of cell functions during development and tissue repair (Larjava et al. 2011). However, little information is available regarding integrin function in ameloblasts during enamel formation. In the present report, we show that $\alpha v\beta 6$ integrin plays a crucial role in enamel biomineralization via regulation of amelogenin and enamelin gene expression.

Integrins likely play key roles in tooth development since several integrins, including $\alpha 6$, αv, $\beta 1$, $\beta 4$ and $\beta 5$ integrin subunits, are expressed in the dental epithelium (Salmivirta et al. 1996). Integrin $\alpha v\beta 5$ may regulate tooth morphogenesis, as its expression oscillates between dental mesenchyme and epithelium (Yamada et al. 1994). Ameloblasts express $\alpha 2\beta 1$ integrin when they assume their columnar shape (Wu and Santoro, 1994), but no tooth phenotype was observed in $\alpha 2$ integrin knockout mice (unpublished data from our laboratory). Loss-of-function mutations in either $\alpha 6$ or $\beta 4$ integrin cause human junctional epidermolysis bullosa, in which the epithelium detaches from the basement membrane causing skin or mucosal blistering (Wright 2010). In these patients, ameloblast adhesion to developing enamel is also reduced, leading to hypoplastic enamel (Wright 2010).

Only a few potential integrin-binding ligands have been identified in the enamel matrix. Amelogenin (Snead et al. 1983) and amelotin (Iwasaki et al. 2005; Moffatt et al. 2006) do not possess RGD motifs or other known integrin recognition sequences. The RGD motif in enamelin is not evolutionarily conserved (Hu et al. 1997; Nawfal et al. 2007). Ameloblastin contains binding sites for the RGD motif-binding integrins (Cern et al. 1996), and it has been shown to bind to ameloblasts, but the receptor has not yet been identified (Fukumoto et al. 2004). Ameloblasts also express bone sialoprotein that has binding sites for both hydroxyapatite and RGD-binding integrins (Ganss et al. 1999; Harris et al. 2000; Stubbs et al. 1997). Dentin sialoprotein, which also contains the RGD motif, is only transiently expressed in presecretory ameloblasts and may contribute to formation of the dentino-enamel junction rather than to enamel maturation (Bgue-Kirn et al. 1998; Paine et al. 2005).

EMD enhances the adhesion, proliferation and matrix production of fibroblast-like but not epithelial cells (Cattaneo et al. 2003; Gestrelius et al. 1997; Haase and Bartold, 2001; Hoang et al. 2000; Kawase et al. 2000). Attachment of human periodontal ligament cells to EMD is possibly mediated by bone sialoprotein as it can be inhibited by RGD-containing peptides or with an anti-$\alpha v\beta 3$ integrin antibody (Suzuki et al. 2001). Also $\beta 1$ inte-

grins seem important to cell adhesion to EMD (van der Pauw et al. 2002). More recently, it was demonstrated that both β1 and αv integrins mediate periodontal ligament fibroblast adhesion to EMD (Narani et al. 2007). Because amelogenins do not contain any known recognition sites for integrins, it is possible that self-aggregation of amelogenins exposes cryptic integrin recognition sites. Consistently with this hypothesis, amelogenins promote binding of fibroblasts and endothelial cells via multiple integrins, including αvβ3, αvβ5 and αvβ1 (Almqvist et al. 2010; Almqvist et al. 2011). In the present study, ameloblast αvβ6 integrin did not directly bind to EMD but the cells attached via endogenously deposited matrix. Some of these matrix molecules appeared to be αvβ6 integrin ligands, such as fibronectin that readily binds to amelogenin (Narani et al. 2007). However, also *Itgb6-/-* ameloblasts were able to attach and spread on this matrix, suggesting collaboration by other integrin receptors in ameloblast adhesion. It appears unlikely that αvβ6 integrin is essential for ameloblast cell adhesion in vivo either, as the ameloblasts retained their columnar shape and adhesion to the developing enamel in the *Itgb6-/-* mice.

Amelogenin or its fragments are endocytosed by ameloblasts via a receptor-mediated mechanism involving LAMP1 and CD63 proteins (Shapiro et al. 2007). This uptake may serve as a feedback loop to upregulate amelogenin expression through stabilization of its mRNA in the cytoplasm (Xu et al. 2006). We did not find evidence for the direct involvement of αvβ6 integrin in amelogenin endocytosis. However, we found that expression of several RBMs (*Rbm26*, *Rbm45, Rbm8a*) was increased (4.2-5-fold) in the enamel organs of *Itgb6-/-* mice compared to WT. RBMs have been shown to critically regulate post-transcriptional RNA metabolism (Janga and Mittal, 2011). Therefore, these proteins could regulate amelogenin and enamelin mRNA stability. It is possible that the lack of αvβ6 integrin signaling in the *Itgb6-/-* ameloblasts may disrupt the feedback system for amelogenin expression and lead to its dysregulated accumulation in the tooth enamel by an as yet unknown mechanism.

The best-known function of αvβ6 integrin is in the activation of latent TGF-β1 (Yang et al. 2007). TGF-β and its receptors are important regulators of early tooth development (Chai et al. 1994; Chai et al. 1999; Pelton et al. 1991). In addition, TGF-β1 is expressed in ameloblasts of developing enamel (Gao et al. 2009). Interestingly, the tooth development in TGF-β1 mutant mice is unaffected, which could be partially due to rescue by maternal TGF-β1 (D'Souza and Litz 1995). TGF-βs binding to type I and II TGF-β receptors (TGFβRI and TGFβRII) induces activation of Smad2/3 (Moustakas and Heldin 2008). Human mutations in TGF-βRI

and II cause Loeys-Dietz syndrome due to abnormal receptor signaling, but no enamel phenotypes have been reported for these patients (Loeys et al., 2005). However, there is evidence that dysregulated TGF-β1 signaling may be detrimental to amelogenesis. Ameloblasts in mice overexpressing TGF-β1 under the dentin sialoprotein promoter show enamel defects, including ameloblast detachment with a pitted and hypoplastic enamel (Haruyama et al. 2006). In K14-*Smad2*-overexpressing mice, the ameloblast layer is disorganized and amelogenin-containing matrix is found between ameloblasts (Ito et al. 2001). No tooth abnormalities have been reported in *Smad7*-/- (an inhibitor of TGF-β signaling) mice. Mice overexpressing *Smad7* under the K5 promoter, however, show suppressed Smad3-mediated signaling and a failure to produce proper enamel (Helder et al. 1998; Klopcic et al. 2007).

In the present study, we did not find evidence for the enamel defects in *Itgb6*-/- *mice*being caused by altered TGF-β1 activation. Neither phosphorylation nor the expression level of Smad3 was altered in the *Itgb6*-/- enamel organ. We also compared histology of enamel from *Smad3*-/- mice to that of *Itgb6*-/-, and they lacked similarity (unpublished results). Previous studies have also shown that ameloblast morphology remains unaltered in *Smad3*-/- mice although the enamel is poorly mineralized, this being likely attributable to defective protein removal at the maturation stage (Yokozeki et al. 2003).

Recently, it was reported that Smad3 and FoxO1 (transcriptional cofactor for Smads) collaboratively regulate genes involved in enamel formation (Poche et al. 2012). Interestingly, in both Smad3 and FoxO1 mutant teeth, the expression of *Ambn*, *Amelx*, *Enam*, *Mmp20* and *Klk4* were all significantly downregulated. The fact that *Amelx* and *Enam* are highly upregulated in β6 integrin mutant teeth also strongly suggests that the observed enamel phenotype is not caused by defective TGF-β1 activation. Clearly, TGF-β signaling regulates enamel formation but significant involvement of αvβ6 integrin in the activation of ameloblast TGF-βs seems unlikely.

We explored the gene expression profiles in the WT and *Itgb6*-/- mouse enamel organs to identify additional changes that could contribute to disturbed mineralization of *Itgb6*-/- mouse enamel. Interestingly, expression of *Ank* was increased in the *Itgb6*-/- enamel organ. ANK functions in transporting inorganic pyrophosphate to the extracellular space, where pyrophosphate serves as a potent inhibitor of extracellular matrix mineralization in calcified tissues (Harmey et al. 2004; Wang et al. 2005; Zaka and Williams 2006). Future studies should therefore investigate whether inorganic pyrophosphate levels are increased in *Itgb6*-/- enamel organs, thereby contributing to the mineralization defect.

Other significant changes in gene expression observed in the *Itgb6*-/-

enamel organ included reduced expression of arrestin *Arrdc3*, photocadherin *Pcdh21* and *Fam20B*. ARRDC3 functions in cell signalling and molecular trafficking (Rajagopal et al. 2010). It remains to be shown whether is involved in the regulation of *Amelx* expression. Mutations in *PCDH21* cause autosomal recessive cone-rod dystrophy in the eye (Ostergaard et al. 2010), which is associated with AI in some patients, thus demonstrating possible links between retinal functions and enamel mineralization (Parry et al. 2009; Polok et al. 2009). Since cell-cell adhesion of ameloblasts via cadherins has been demonstrated to be critical for enamel mineralization (Bartlett et al. 2010), it is possible that down-regulation of *Pcdh21* contributes to weakened ameloblast cell-cell adhesion and subsequent accumulation of amelogenins between the cells. FAM20B is a kinase implicated in phosphorylation and control of proteoglycans (Koike et al. 2009). It and other members of the FAM20 protein family have multiple roles in mineralized tissues. Mutations in *FAM20A* cause hypoplastic AI and gingival overgrowth (Cho et al. 2011; O'Sullivan et al. 2011), whereas FAM20C that is expressed in mineralized tissues, including enamel and dentin phosphorylates secreted mineral-binding proteins and is essential for normal bone development (Hao et al. 2007; Simpson et al. 2007; Wang et al. 2010; Ishikawa et al. 2012). FAM20B is expressed during the maturation stage of amelogenesis (O'Sullivan et al. 2011), but its function in enamel remains unknown.

Amelogenesis imperfecta represents a collection of genetic disorders that affect enamel formation both in the primary and permanent dentition in the absence of systemic manifestations (Hu et al. 2007). Mutations in several genes are associated with human AI (Hu et al. 2007; Stephanopoulos et al. 2005). In general, mutations that affect enamel matrix production (*AMELX*, *AMBN*, *ENAM*) tend to result in hypoplastic enamel while those that affect matrix removal (*MMP20*, *KLK4*) tend to result in hypomaturation of the enamel (Hu et al. 2007). Interestingly, previous studies with overexpression of normal amelogenin or its various fragments have not shown major alterations in enamel structure (Chen et al. 2003; Gibson et al. 2007; Paine et al. 2004). However, the present study clearly demonstrates that the enamel defect resulting from $\alpha v\beta 6$ integrin deficiency and the subsequent overexpression of amelogenin and enamelin is detrimental to enamel formation and leads to a condition that mimics the hypomaturation type of AI. Therefore, *ITGB6* should be considered a candidate gene for human AI with normal thickness but altered prism structure and reduced mineralization. As discussed above, there are several putative mechanisms for how $\alpha v\beta 6$ integrin regulates enamel biomineralization that need to be explored further.

2.4 Materials and methods

2.4.1 Animals

The Animal Care Committee of the University of British Columbia approved all animal procedures used in this study. The mouse lines used were: WT (FVB background), *Itgb6-/-* (a generous gift from Dr. Dean Sheppard, University of California, San Francisco, CA, USA), K14β6 that overexpresses human β6 integrin under the K14 promoter (Häkkinen et al. 2004) and β6 rescue mice representing *Itgb6-/-* mice that have been bred with the K14β6 mice (F1; also from Dr. Sheppard). The mice were maintained in a conventional animal care facility and had free access to standard mouse chow (Purina 5001) and water. Animals were sacrificed by CO_2 inhalation.

2.4.2 Western blotting

The incisors of the maxillas and mandibles of 3-9-month-old mice (three animals, 8-10 incisors per group) were removed, and their enamel organs scraped off with a microsurgical blade and pooled for Western blotting. The antibodies used were: ameloblastin (ab72776; Abcam, Cambridge, MA, USA), amelogenin (pc-062; Kamiya Biomedical Company, Seattle, WA, USA), KLK4 (ab3636; Abcam), MMP20 (ab76109; Abcam), Smad3 (ab28379; Abcam), pSmad3 ab52903; Abcam), β6 integrin (AF2389; R&D Systems, Inc., Minneapolis, MN, USA), αv integrin (sc-6618, Santa Cruz Biotechnology, Santa Cruz, CA, USA), β1 integrin (4080; Larjava et al. 1990), β-actin (ab8227; Abcam) and β-tubulin (mAb3408, Millipore, Temencula, CA, USA). Appropriate peroxidase-conjugated IgGs (Santa Cruz) were used as secondary antibodies. After washing, the protein bands were detected using ECL Western Blotting Detection Kit (GE Healthcare, Baie d'Urfe, QC, Canada), and the digitized images were quantified using the ImageJ software (available at http://rsb.info.nih.gov/ij; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). Alternatively, IRDye-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) were used and the blots analyzed and quantitated with Odyssey infrared reader (LI-COR).

2.4.3 Gene expression profiling by microarray

The enamel organs were collected from the incisors of 6-month-old WT and *Itgb6-/-* mice, pooled (three animals, 8-10 incisors per group), placed into RNAlater (Ambion, Life Technologies, Inc., Burlington, ON, Canada) and

stored at -80°C. Total RNA was extracted using either NucleoSpin RNA II or XS kit (Macherey-Nagel, Bethlehem, PA, USA) and treated with DNaseI digestion. RNA samples were analyzed using Illumina Mouse WG-6 v.2.0 Expression BeadChip at the Finnish Microarray and Sequencing Centre (Turku Centre for Biotechnology, Turku, Finland) followed by data analysis with R (R Development Core Team 2008) and Bioconductor (Gentleman et al. 2004) softwares. The data were quantile normalized, and statistical analysis for detecting the global differences in gene expression between the groups was carried out using Bioconductor's Limma package. The chosen thresholds used in filtering the differentially expressed genes were FDR p-value<0.05 and absolute fold change >1.6 as the comparison cutoff.

2.4.4 RNA analysis by PCR

Total RNA (0.5 μg) was reverse-transcribed with SuperScript VILO cDNA Synthesis Kit (Invitrogen, Life Technologies). Real-time PCR amplification was performed on the MiniOpticon Real-Time System (Bio-Rad, Mississauga, ON, Canada) using 5 μl of RT products (diluted to a concentration where the C_T values were well within the range of the standard curve) mixed with 10 μl of 2x iQ SYBR Green I Supermix (Bio-Rad) and 5 pmoles of primers, in a final volume of 20 μl. An amplification reaction was conducted for target genes with *Actb*, *Gapdh* and *Hprt1* as reference genes and replicated 9 times for each sample. The data were analyzed based on the comparative C_T program of Gene Expression Analysis for iCycler iQ Real-Time PCR Detection System (Bio-Rad). Primer sequences (5'-3') were: *Actb* (CTTCCTTCTTGGGTATGGAATC, TAGAGGTCTTTACGGATGTCAAC), *Ambn* (CTTCCTTCTTGGGTATGGAATC, TAGAGGTCTTTACGGATGTCAAC), *Amelx* (GCTTTTGCTATGCCCCTA, CTCATAGCTTAAGTTGATATAACCA), *Itgb6* (AATCACCAACCCTTGCAGTAG, AATGTGCTTGAATCCAAATGTAG), *Enam* (TCTCTGCTGCCATGCCATTC, TTGATTATATCGCATCATCTCTTCAC), *Klk4* (CATCCCTGTGGCTACCCAA, GGGCAGTTTCCCATTCTTTA), *Mmp20* (TGCTGTGGAACTGAATGGCTA, ACACTAACCACGTCTTCCTTC), *Hprt1* (TGTTGGATTTGAAATTCCAGACAAG, CTTTTCCAGTTTCACTAATGACACAA), *Krt14* (ATCCTCTCAATTCTCCTCTGGCTC, ACCTTGCCATCGTGCACATC), *Itgb1* (GCTGGTTCTATTTCACCTATTCA, CAACCACGCCTGCTACAA), *Itgb4* (CCAGCTGAGACCAATGGCGA, GAGCACCTTCTTCATAGGTCCA, *Gapdh* (CTTTGTCAAGCTCATTTCCTGGTA, GGCCATGAGGTCCACCA).

2.4.5 Attrition rate

Maxillas and mandibles of 3-24-month-old WT and *Itgb6-/-* mice were defleshed mechanically and with 2% KOH (EM Science, Merck, Darmstadt, Germany). Attrition of the molars was scored based on cusp heights where 0= <10% attrition of the cusps, 1= attrition reaching up to 50% of the cusp height, and 2= more than 50% attrition. In a cross-sectional study, we also compared the attrition rate of molars in 6-month-old WT, *Itgb6-/-*, K14-β6 and rescue mice.

2.4.6 Scanning electron microscopy (SEM)

Calcium-to-phosphorus ratio

WT and *Itgb6-/-* mice (11-14-month-old) were sacrificed, decapitated and their heads were fixed in 4% formaldehyde in phosphate-buffered saline (PBS, pH 7.2). The mandibles were dissected and further fixed in 2.5% glutaraldehyde for 1 h at 4°C. The samples were post-fixed with 1 M OsO_4 for 1 h at RT. After three rinses, the specimens were dehydrated, critical-point dried, gold-coated and viewed by SEM. Three standardized areas (mesial, middle and distal) on the mid-lingual surface of the third molar were used to measure calcium-to-phosphorus ratios using energy dispersive X-ray spectroscopy (Hitachi S-3000N SEM with light element EDX).

Visualization of prism structures

Defleshed and dried incisors from WT and *Itgb6-/-* mice were frozen by immersion in liquid nitrogen and then fractured (along the line from root to crown) with a pre-cooled scalpel blade. The fractured portion of the incisor was treated at RT in 35% phosphoric acid for 30 s, rinsed and air-dried (Snchez-Quevedo et al. 2006). The incisors were then mounted and coated with a 10 nm layer of gold/palladium. Each sample was imaged by SEM with identical imaging conditions at 4 kV.

2.4.7 Immunohistochemistry

Immunostaining of β6 integrin was performed on frozen sections (6 μm) of the secretory stage of an upper incisor and a developing upper molar from a 3-day-old WT mouse using the β6 integrin antibody β6B1 (a generous gift from Dr. Dean Sheppard).

Impacted human third molars containing the dental follicle were collected anonymously from patients requiring extractions of these teeth as a

part of their treatment (approved by the Clinical Research Ethics Board, University of British Columbia). Teeth with soft tissue remaining were fixed with 2% formaldehyde in PBS and then decalcified in the same fixative containing 0.4 M EDTA. Frozen sections (6 μm) containing patches of cells resembling ameloblasts were identified and used for immunolocalization of K14 (MCA890; AbD Serotec, Oxford, UK), amelogenin and β6 integrin as described above using a fluorescently-labeled secondary antibody (Alexa 488; Invitrogen).

2.4.8 Mineral analysis of incisors

Hemi-maxillas and -mandibles from 7-week-old male and female WT and *Itgb6-/-* mice were removed and cleaned of adhering soft tissues. Procedures employed for isolating and removing sequential 1-mm-long strips of developing enamel from maxillary and mandibular incisors have been described in detail previously (Smith et al. 2005; Smith et al. 2009; Smith et al. 2011). Directly measured 'before' and 'after' heating weights were used to calculate various parameters including mineral-to-volatiles ratio, which represents the after-heating weight (mineral content of sample) divided by the difference between the initial dry weight minus the after-heating weight (amount of volatiles in sample; mostly protein). Weight data for enamel strips were collected from a minimum of 9 maxillary and 9 mandibular samples per genotype.

2.4.9 In situ hybridization

In situ hybridization using digoxigenin-UTP labeled riboprobes was carried out as previously described (Yoshida et al. 2010). A plasmid for integrin β6 integrin riboprobe was generated by inserting PCR products from mouse cDNA into pCRII-TOPO vector (Invitrogen). Primers used for the PCR were aggggtgactgctattgtgg and ggcaccaatgctttacact.

2.4.10 Undecalcified histology, transmission electron microscopy and immunogold labeling

WT and *Itgb6-/-* mouse mandibles were immersion-fixed overnight in sodium cacodylate-buffered aldehyde solution and cut into segments containing the molars, underlying incisor and surrounding alveolar bone. The samples were dehydrated through a graded ethanol series and infiltrated in acrylic resin (LR White; London Resin Company, Berkshire, UK) followed by polymerization of at 55° C for two days. 1-μm sections of hemi-mandibles were

cut with a diamond knife using an ultramicrotome, and glass slide-mounted sections were stained with 1% toluidine blue and von Kossa reagent for mineral. Immunoglod-labeling for amelogenin coupled with TEM was performed as described previously (McKee and Nanci 1995; McKee et al. 1996) using an anti-amelogenin antibody kindly provided by Dr. Takashi Uchida of Hiroshima University, Japan (Uchida et al. 1991).

2.4.11 Micro-computed tomography

Micro-computed tomography (Micro-CT, model 1072; Skyscan, Kontich, Belgium) of undecalcified hemi-mandibles was performed at the level of the first molar from three samples of each genotype. The X-ray source was operated at maximum power (80 KeV) and at 100 μA. Images were captured using a 12-bit, cooled, charge-coupled device camera (1024×1024 pixels) coupled by a fiber optic taper to the scintillator. Using a rotation step of 0.9, total scanning time was 35 min for each sample with a scan resolution of 5 μm, after which ∼300 sections (slice-to-slice distance of 16.5 μm) were reconstructed using Skyscan tomography software. Appropriate imaging planes were selected to show three-dimensional longitudinal and cross-sectional 'sections' (segments) of the first molar and underlying incisor.

2.4.12 Establishment of ameloblast cell lines

Maxillary and mandibular incisors of 5-6-month-old WT and *Itgb6-/-* mice were prepared under aseptic conditions and placed in cell culture wells precoated with bovine fibronectin (10 μg/ml; Millipore) and collagen (30 μg/ml; PureCol®; Advanced BioMatrix, Inc., San Diego, CA, USA) in keratinocyte growth medium (KCM; Häkkinen et al. 2001). The cells growing out from the explants were differentially trypsinized to selectively remove the more easily released fibroblastic cells. The remaining epitheloid cells were confirmed to express ameloblast marker K14 as well as to be positive (WT) or negative (*Itgb6-/-*) for β6 integrin expression by PCR. The ameloblasts were routinely grown in KCM.

2.4.13 Cell spreading assays

For cell spreading assays, WT ameloblasts were seeded in plates coated with EMD (10,000 μg/ml in 10 mM acetic acid; Emdogain® Straumann, Basel, Switzerland) in triplicates in the presence or absence of 50 μM cycloheximide

(a protein synthesis blocker) and 200 nM TPA (an integrin activator). Cell spreading was quantified as described previously (Narani et al., 2007).

To explore the role of $\alpha v\beta 6$ integrin in cell spreading on EMD, WT or *Itgb6-/-* ameloblasts were pre-incubated on ice with anti-integrin antibodies and then allowed to spread on EMD in the presence of TPA and in the absence of cycloheximide. The anti-$\alpha v\beta 6$ integrin antibodies used were: MAB2077Z (50 μg/ml; Millipore), 6.8G6 (10 μg/ml; Biogen Idec, Cambridge, MA, USA; Weinreb et al. 2004) and 6.3G9 (10 μg/ml; Biogen).

2.4.14 Amelogenin endocytosis by ameloblast-like cells

WT and *Itgb6-/-* ameloblasts were seeded onto glass coverslips (10,000 cells per ml) for 24 h in KCM. The cells were treated with KCM only, with 100 μg/ml of EMD or pre-treated for 10 min with 20 μM E64d [(2S,3S)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester; inhibits lysosomal degradation of endocytosed proteins; McGowan et al. 1989)], followed by addition of 100 μg/ml of EMD. The cells were then incubated for 24 h and processed for immunofluorescence staining with anti-amelogenin antibody (PC-062).

2.4.15 Statistical analysis

The experiments were repeated at least three times. The difference between WT and *Itgb6-/-* mice was calculated using unpaired, two-tailed Student's t-test using GraphPad InStat 3 software. Multiple comparison tests were performed using one-way ANOVA with Tukey's post-test. Statistical significance was set at $p<0.05$.

Chapter 3

Conclusion and future studies

In this paper, we demonstrate for the first time that ameloblast cell surface $\alpha v\beta 6$ integrin receptor critically regulates enamel biomineralization via regulation of amelogenin and enamelin gene expression. In addition, we show that $\beta 6$ integrin deficiency leads to amelogenesis imperfecta-like phenotype in mouse dentition. These mice show severe enamel defects including reduced biomineralization of enamel and altered surface structure. Previously, it was reported that the secretome of rat incisor enamel organ contains $\beta 6$ integrin transcript, but its function in enamel formation has not been previously elucidated (Moffatt et al. 2006).

Integrin $\alpha v\beta 6$ is an exclusively epithelial adhesion protein that is absent from most parts of normal healthy epidermis and oral mucosa (Breuss et al. 1993). However, recent studies indicate that $\alpha v\beta 6$ integrin is constitutively expressed in the junctional epithelium and oral epithelium of the gingival papilla (Csiszar et al. 2007; Ghannad et al. 2008). In vitro, $\alpha v\beta 6$ integrin binds to Arg-Gly-Asp (RGD) sequence containing extracellular matrix ligands. Amelogenin and most of the enamel matrix proteins (except bone and dentin sialoproteins) do not posses RGD motifs (Snead et al. 1983; Hu et al. 1997; Stubbs et al. 1997; Chen et al. 1998; Bgue-Kirn et al. 1998; Ganss et al. 1999; Harris et al. 2000; Paine et al. 2005; Iwasaki et al. 2005; Moffatt et al. 2006; Nawfal et al. 2007), making it unlikely that the ameloblast $\alpha v\beta 6$ integrins would directly interact with these molecules. However, there is the possibility that there are cryptic sites within the matrix proteins that could unveil a ligand binding region which can interact with the $\alpha v\beta 6$ receptor. This could lead to binding of enamel matrix protein to the $\alpha v\beta 6$ receptor. There is also the possibility of bridging molecules that can act as a liason to allow matrix proteins to bind to the $\alpha v\beta 6$ integrin. There may be bridging molecules that contain the RGD motif as well as a binding region that allows interaction of the matrix proteins with the $\alpha v\beta 6$ integrin in an indirect manner, such as fibronectin (Narani et al. 2007).

Our data indicate that $\beta 6$ integrin deficiency leads to accumulation of

amelogenins in the enamel matrix leading to the mineralization defect and subsequent enamel attrition. Moreover, there is an increased expression of amelogenins and enamelin in their enamel organ. We also showed that this accumulation is likely due to excess production rather than reduced degradation of amelogenin.

Future studies should determine the relation between presence of this integrin and gene expression of enamel matrix proteins such as amelogenin. First, cell lines that have an increased amelogenin expression should be identified. $\alpha v\beta 6$ integrin should be blocked using either antibodies or SiRNA. Then signaling pathways and transcription factors should be studied.

Amelogenin or its fragments are endocytosed by ameloblasts (Shapiro et al. 2007). This uptake may serve as a mechanism to regulate the amelogenin expression (Xu et al. 2006). It is therefore possible that $\alpha v\beta 6$ integrin is involved in the regulation of endocytosis of peptides from enamel protein, and lack of the integrin may lead to dysregulated accumulation and expression of amelogenins in the enamel. When we seeded the WT and $\beta 6$ integrin knockout ameloblasts in EMD coated (amelogenin rich) wells, there was no difference in the pattern of endocytosis between the two cell lines. However, in vivo, amelogenin is surrounded by degradative enzymes such as MMP20 and Klk4 which break down amelogenin into smaller fragments, and most likely it is these breakdown products that are actually endocytosed by the ameloblasts. A future study would be needed that includes seeding the ameloblast cells (WT and $\beta 6$ integrin knockout) on a matrix that is more representative of the in vivo matrix found surrounding ameloblasts. The matrix should include amelogenin along with the various degradative enzymes, which cause in the formation of the amelogenin fragments, and the uptake of these fragments can be compared between the two cell lines. This type of study may show that $\alpha v\beta 6$ integrin does in fact disrupt the endocytosis and the feedback system for amelogenin expression.

Our cell-spreading experiment results further indicated that the $\alpha v\beta 6$ integrin has an indirect role in the adhesion of ameloblast cells to amelogenin-rich matrix, and that the cells attached via an endogenously deposited matrix. It thus seems that the $\alpha v\beta 6$ integrin is not essential for the ameloblast cell adhesion as the $\alpha v\beta 6$ integrin knockout cells were also able to attach to the matrix in vitro, and the ameloblast cells remained attached to the developing enamel in the knockout mice in vivo.

Ameloblasts express TGF-$\beta 1$, which contains the RGD site in the latent molecule (Gao et al. 2009). TGF-$\beta 1$ is a cell-signaling molecule that has an important role in regulating tooth development during the early stages (Chai et al. 1994; Chai et al. 1999; Pelton et al. 1991). Binding of $\alpha v\beta 6$

integrin to the RGD site in the latent TGF-β1 causes activation of this potent cytokine (Munger et al. 1999; Annes et al. 2004). Activated TGF-β1 in turn phosphorylates Smad2 and Smad3 which mediate the signals to the nucleus (Yokozeki et al. 2003). The results of our study indicate that enamel defects in the knockout mice is unlikely to be caused by altered TGF-β1 activation, as no change was detected in the phosphorylation and expression level of Smad2/3 in the β6-/- mice enamel organ. Interestingly however, smad3-/- mice share similarities in enamel defects with the β6-/- mice (Yokozeki et al. 2003; Brown et al., 2007). It is clear that the TGF-β signaling regulates the amelogenesis process, but the role of αvβ6 integrin in activation of TGF-βs in the ameloblast does not seem to be significant. The exact mechanism and intracellular signaling leading to the enamel defects in the β6-/- mice remain to be further investigated.

Lastly, there were other significant changes in the gene expression of β6-/- mice enamel organ that included an increased expression of Ank, and reduced expression of arrestin domain containing 3 protein (Arrdc2), photocadherin21 (Pdch21), and family with sequence homology 20, member B (Fam20B). These genes have different roles in cell-adhesion and mineralization, and regulation of these genes in the β6-/- mice enamel organ may contribute to the hypomineralized enamel phenotype. Future studies should investigate the role of these genes in the enamel formation process. Futures studies should also further focus on investigating the mechanisms through which the deficiency of this integrin leads to the observed abnormalities and clarify whether mutations in the β6 integrin gene is associated with either human genetic (amelogenesis imperfecta) or acquired conditions that affect enamel formation.

Bibliography

[1] J. T. Stubbs 3rd, K. P. Mintz, E. D. Eanes, D. A. Torchia, and L. W. Fisher. Characterization of native and recombinant bone sialoprotein: delineation of the mineral-binding and cell adhesion domains and structural analysis of the rgd domain. *Journal of bone and mineral research*, 12(8):1210–1222, 1997.

[2] H. Akin, S. Tasveren, and D. Y. Yeler. Interdisciplinary approach to treating a patient with amelogenesis imperfecta: a clinical report. *Journal of esthetic and restorative dentistry*, 19(3):131–5; discussion 136, 2007.

[3] M. J. Aldred and P. J. Crawford. Amelogenesis imperfecta–towards a new classification. *Oral diseases*, 1(1):2–5, 1995.

[4] M. J. Aldred, P. J. Crawford, E. Roberts, and N. S. Thomas. Identification of a nonsense mutation in the amelogenin gene (amelx) in a family with x-linked amelogenesis imperfecta (aih1). *Human genetics*, 90(4):413–416, 1992.

[5] M. J. Aldred, R. Savarirayan, and P. J. Crawford. Amelogenesis imperfecta: a classification and catalogue for the 21st century. *Oral diseases*, 9(1):19–23, 2003.

[6] S. Almqvist, M. Werthen, A. Johansson, M. S. Agren, P. Thomsen, and S. P. Lyngstadaas. Amelogenin is phagocytized and induces changes in integrin configuration, gene expression and proliferation of cultured normal human dermal fibroblasts. *Journal of materials science.Materials in medicine*, 21(3):947–954, 2010.

[7] S. Almqvist, M. Werthen, S. P. Lyngstadaas, C. Gretzer, and P. Thomsen. Amelogenins promote an alternatively activated macrophage phenotype in vitro. *International Journal of Nano and Biomaterials*, 3:282–298, 2011.

[8] P. Aluwihare, Z. Mu, Z. Zhao, D. Yu, P. H. Weinreb, G. S. Horan, S. M. Violette, and J. S. Munger. Mice that lack activity of alphavbeta6- and alphavbeta8-integrins reproduce the abnormalities of tgfb1- and tgfb3-null mice. *Journal of cell science*, 122(Pt 2):227–232, 2009.

[9] J. P. Annes, Y. Chen, J. S. Munger, and D. B. Rifkin. Integrin alphavbeta6-mediated activation of latent tgf-beta requires the latent tgf-beta binding protein-1. *The Journal of cell biology*, 165(5):723–734, 2004.

[10] J. P. Annes, D. B. Rifkin, and J. S. Munger. The integrin alphavbeta6 binds and activates latent tgfbeta3. *FEBS letters*, 511(1-3):65–68, 2002.

[11] B. Backman. Inherited enamel defects. *Ciba Foundation symposium*, 205:175–82; discussion 183–6, 1997.

[12] B. Backman and A. K. Holm. Amelogenesis imperfecta: prevalence and incidence in a northern swedish county. *Community dentistry and oral epidemiology*, 14(1):43–47, 1986.

[13] B. Backman and G. Holmgren. Amelogenesis imperfecta: a genetic study. *Human heredity*, 38(4):189–206, 1988.

[14] M. Barczyk, S. Carracedo, and D. Gullberg. Integrins. *Cell and tissue research*, 339(1):269–280, 2010.

[15] J. D. Bartlett, E. Beniash, D. H. Lee, and C. E. Smith. Decreased mineral content in mmp-20 null mouse enamel is prominent during the maturation stage. *Journal of dental research*, 83(12):909–913, 2004.

[16] J. D. Bartlett, J. M. Dobeck, C. E. Tye, M. Perez-Moreno, N. Stokes, A. B. Reynolds, E. Fuchs, and Z. Skobe. Targeted p120-catenin ablation disrupts dental enamel development. *PloS one*, 5(9):e12703, 2010.

[17] J. D. Bartlett, J. P. Simmer, J. Xue, H. C. Margolis, and E. C. Moreno. Molecular cloning and mrna tissue distribution of a novel matrix metalloproteinase isolated from porcine enamel organ. *Gene*, 183(1-2):123–128, 1996.

[18] M. J. Beanan and T. D. Sargent. Regulation and function of dlx3 in vertebrate development. *Developmental Dynamics*, 218:545–553, 2000.

[19] C. Begue-Kirn, P. H. Krebsbach, J. D. Bartlett, and W. T. Butler. Dentin sialoprotein, dentin phosphoprotein, enamelysin and ameloblastin: tooth-specific molecules that are distinctively expressed during murine dental differentiation. *European journal of oral sciences*, 106(5):963–970, 1998.

[20] M. Bei. Molecular genetics of ameloblast cell lineage. *Journal of experimental zoology.Part B, Molecular and developmental evolution*, 312B(5):437–444, 2009.

[21] M. Bei, S. Stowell, and R. Maas. Msx2 controls ameloblast terminal differentiation. *Developmental dynamics* , 231(4):758–765, 2004.

[22] D. Bouvard, C. Brakebusch, E. Gustafsson, A. Aszodi, T. Bengtsson, A. Berna, and R. Fassler. Functional consequences of integrin gene mutations in mice. *Circulation research*, 89(3):211–223, 2001.

[23] P. Bouwman, H. Gollner, H. P. Elsasser, G. Eckhoff, A. Karis, F. Grosveld, S. Philipsen, and G. Suske. Transcription factor sp3 is essential for post-natal survival and late tooth development. *The EMBO journal*, 19(4):655–661, 2000.

[24] J. M. Breuss, J. Gallo, H. M. DeLisser, I. V. Klimanskaya, H. G. Folkesson, J. F. Pittet, S. L. Nishimura, K. Aldape, D. V. Landers, and W. Carpenter. Expression of the beta 6 integrin subunit in development, neoplasia and tissue repair suggests a role in epithelial remodeling. *Journal of cell science*, 108 (Pt 6)(Pt 6):2241–2251, 1995.

[25] J. M. Breuss, N. Gillett, L. Lu, D. Sheppard, and R. Pytela. Restricted distribution of integrin beta 6 mrna in primate epithelial tissues. *The journal of histochemistry and cytochemistry*, 41(10):1521–1527, 1993.

[26] J. M. Breuss, N. Gillett, L. Lu, D. Sheppard, and R. Pytela. Restricted distribution of integrin β6 mrna in primate epithelial tissues. 41:1521–1527, 1993.

[27] S. J. Brookes, C. Robinson, J. Kirkham, and W. A. Bonass. Biochemistry and molecular biology of amelogenin proteins of developing dental enamel. *Archives of Oral Biology*, 40(1):1–14, 1995.

[28] K. A. Brown, J. A. Pietenpol, and H. L. Moses. A tale of two proteins: differential roles and regulation of smad2 and smad3 in tgf-beta signaling. *Journal of cellular biochemistry*, 101(1):9–33, 2007.

[29] K. Burridge and M. Chrzanowska-Wodnicka. Focal adhesions, contractility, and signaling. *Annual Review of Cell and Developmental Biology*, 12:463–518, 1996.

[30] M. Busk, R. Pytela, and D. Sheppard. Characterization of the integrin alpha v beta 6 as a fibronectin-binding protein. *The Journal of biological chemistry*, 267(9):5790–5796, 1992.

[31] I. D. Campbell and M. J. Humphries. Integrin structure, activation, and interactions. *Cold Spring Harbor perspectives in biology*, 3(3):10.1101/cshperspect.a004994, 2011.

[32] J. J. Caterina, Z. Skobe, J. Shi, Y. Ding, J. P. Simmer, H. Birkedal-Hansen, and J. D. Bartlett. Enamelysin (matrix metalloproteinase 20)-deficient mice display an amelogenesis imperfecta phenotype. *The Journal of biological chemistry*, 277(51):49598–49604, 20 2002.

[33] V. Cattaneo, C. Rota, M. Silvestri, C. Piacentini, A. Forlino, A. Gallanti, G. Rasperini, and G. Cetta. Effect of enamel matrix derivative on human periodontal fibroblasts: proliferation, morphology and root surface colonization. an in vitro study. *Journal of periodontal research*, 38(6):568–574, 2003.

[34] R. Cerny, I. Slaby, L. Hammarstrom, and T. Wurtz. A novel gene expressed in rat ameloblasts codes for proteins with cell binding domains. *Journal of bone and mineral research*, 11(7):883–891, 1996.

[35] Y. Chai, A. Mah, C. Crohin, S. Groff, P. Bringas Jr, T. Le, V. Santos, and H. C. Slavkin. Specific transforming growth factor-beta subtypes regulate embryonic mouse meckel's cartilage and tooth development. *Developmental biology*, 162(1):85–103, 1994.

[36] Y. Chai, J. Zhao, A. Mogharei, B. Xu, P. Bringas Jr, C. Shuler, and D. Warburton. Inhibition of transforming growth factor-beta type ii receptor signaling accelerates tooth formation in mouse first branchial arch explants. *Mechanisms of development*, 86(1-2):63–74, 1999.

[37] H-C Chan, L. Mai, A. Oikonomopoulou, H. L. Chan, A. S. Richardson, S-K Wang, J. P. Simmer, and J. C-C Hu. Altered enamelin phosphorylation site causes amelogenesis imperfecta. *J.Dent.Res.*, 89(7):695–699, 2010.

[38] E. Chen, Z. A. Yuan, J. T. Wright, S. P. Hong, Y. Li, P. M. Collier, B. Hall, M. D'Angelo, S. Decker, R. Piddington, W. R. Abrams, A. B. Kulkarni, and C. W. Gibson. The small bovine amelogenin lrap fails to rescue the amelogenin null phenotype. *Calcified tissue international*, 73(5):487–495, 2003.

[39] J. Chen, K. Sasaguri, J. Sodek, T. B. Aufdemorte, H. Jiang, and H. F. Thomas. Enamel epithelium expresses bone sialoprotein (bsp). *European journal of oral sciences*, 106 Suppl 1:331–336, 1998.

[40] S. H. Cho, F. Seymen, K. E. Lee, S. K. Lee, Y. S. Kweon, K. J. Kim, S. E. Jung, S. J. Song, M. Yildirim, M. Bayram, E. B. Tuna, K. Gencay, and J. W. Kim. Novel fam20a mutations in hypoplastic amelogenesis imperfecta. *Human mutation*, 33(1):91–94, 2012.

[41] Y. H. Chun, Y. Yamakoshi, F. Yamakoshi, M. Fukae, J. C. Hu, J. D. Bartlett, and J. P. Simmer. Cleavage site specificity of mmp-20 for secretory-stage ameloblastin. *Journal of dental research*, 89(8):785–790, 2010.

[42] R. A. Clark, G. S. Ashcroft, M. J. Spencer, H. Larjava, and M. W. Ferguson. Re-epithelialization of normal human excisional wounds is associated with a switch from alpha v beta 5 to alpha v beta 6 integrins. *The British journal of dermatology*, 135(1):46–51, 1996.

[43] P. M. Collier, J. J. Sauk, S. J. Rosenbloom, Z. A. Yuan, and C. W. Gibson. An amelogenin gene defect associated with human x-linked amelogenesis imperfecta. *Archives of Oral Biology*, 42(3):235–242, 1997.

[44] N. Crampton, N. H. Thomson, J. Kirkham, C. W. Gibson, and W. A. Bonass. Imaging rna polymerase-amelogenin gene complexes with single molecule resolution using atomic force microscopy. *European journal of oral sciences*, 114 Suppl 1:133–8; discussion 164–5, 380–1, 2006.

[45] P. J. Crawford, M. Aldred, and A. Bloch-Zupan. Amelogenesis imperfecta. *Orphanet journal of rare diseases*, 2:17, 2007.

[46] P. J. Crawford and M. J. Aldred. X-linked amelogenesis imperfecta. presentation of two kindreds and a review of the literature. *Oral surgery, oral medicine, and oral pathology*, 73(4):449–455, 1992.

[47] S. E. Crawford, V. Stellmach, J. E. Murphy-Ullrich, S. M. Ribeiro, J. Lawler, R. O. Hynes, G. P. Boivin, and N. Bouck. Thrombospondin-1 is a major activator of tgf-beta1 in vivo. *Cell*, 93:1159–1170, 1998.

[48] A. Csiszar, C. Wiebe, H. Larjava, and L. Hakkinen. Distinctive molecular composition of human gingival interdental papilla. *Journal of periodontology*, 78(2):304–314, 2007.

[49] S. L. Dallas, S. Park-Synder, K. Miyazono, D. Twardzik, G. R. Mundy, and L. F. Bonewald. Characterization and autoregulation of latent transforming growth factor beta (tgf beta) complexes in osteoblast-like cell lines. production of a latent complex lacking the latent tgf beta-binding protein. *J.Biol.Chem.*, 269:6815–6821, 1994.

[50] J. C. de Vicente, R. Cabo, E. Ciriaco, R. Laura, F. J. Naves, I. Silos-Santiago, and J. A. Vega. Impaired dental cytodifferentiation in glial cell-line derived growth factor (gdnf) deficient mice. *Annals of Anatomy = Anatomischer Anzeiger*, 184(1):85–92, 2002.

[51] M. Deakins and J. F. Volker. Amount of organic matter in enamel from several types of human teeth. *J.Dent.Res.*, 20:117–121, 1941.

[52] D. Deutsch. Structure and function of enamel gene products. *The Anatomical Record*, 224(2):189–210, 1989.

[53] Y. Ding, M. R. Estrella, Y. Y. Hu, H. L. Chan, H. D. Zhang, J. W. Kim, J. P. Simmer, and J. C. Hu. Fam83h is associated with intracellular vesicles and adhcai. *Journal of dental research*, 88(11):991–996, 2009.

[54] R. Dobrowolski, P. Sasse, J. W. Schrickel, M. Watkins, J. S. Kim, M. Rackauskas, C. Troatz, A. Ghanem, K. Tiemann, J. Degen, F. F. Bukauskas, R. Civitelli, T. Lewalter, B. K. Fleischmann, and K. Willecke. The conditional connexin43g138r mouse mutant represents a new model of hereditary oculodentodigital dysplasia in humans. *Human molecular genetics*, 17(4):539–554, 2008.

[55] J. Dong, D. Amor, M. J. Aldred, T. Gu, M. Escamilla, and M. MacDougall. Dlx3 mutation associated with autosomal dominant amelogenesis imperfecta with taurodontism. *American journal of medical genetics.Part A*, 133A(2):138–141, 1 2005.

[56] J. Dong, T. T. Gu, D. Simmons, and M. MacDougall. Enamelin maps to human chromosome 4q21 within the autosomal dominant amelogenesis imperfecta locus. *European journal of oral sciences*, 108(5):353–358, 2000.

[57] K. M. Draheim, H. B. Chen, Q. Tao, N. Moore, M. Roche, and S. Lyle. Arrdc3 suppresses breast cancer progression by negatively regulating integrin beta4. *Oncogene*, 29(36):5032–5047, 2010.

[58] R. N. D'Souza and M. Litz. Analysis of tooth development in mice bearing a tgf-beta 1 null mutation. *Connective tissue research*, 32(1-4):41–46, 1995.

[59] C. M. Dubois, M. H. Laprise, F. Blanchette, L. E. Gentry, and R. Leduc. Processing of transforming growth factor beta 1 precursor by human furin convertase. *The Journal of biological chemistry*, 270(18):10618–10624, 1995.

[60] B. R. DuPont, C. C. Hu, X. Reveles, and J. P. Simmer. Assignment of serine protease 17 (prss17) to human chromosome bands 19q13.3–¿q13.4 by in situ hybridization. *Cytogenetics and cell genetics*, 86(3-4):212–213, 1999.

[61] B. F. Eames, Y. L. Yan, M. E. Swartz, D. S. Levic, E. W. Knapik, J. H. Postlethwait, and C. B. Kimmel. Mutations in fam20b and xylt1 reveal that cartilage matrix controls timing of endochondral ossification by inhibiting chondrocyte maturation. *PLoS genetics*, 7(8):e1002246, 2011.

[62] J. E. Eastoe. Enamel protein chemistry–past, present and future. *Journal of dental research*, 58(Spec Issue B):753–764, 1979.

[63] W. El-Sayed, D. A. Parry, R. C. Shore, M. Ahmed, H. Jafri, Y. Rashid, S. Al-Bahlani, S. Al Harasi, J. Kirkham, C. F. Inglehearn, and A. J. Mighell. Mutations in the beta propeller wdr72 cause autosomal-recessive hypomaturation amelogenesis imperfecta. *American Journal of Human Genetics*, 85(5):699–705, 2009.

[64] W. El-Sayed, R. C. Shore, D. A. Parry, C. F. Inglehearn, and A. J. Mighell. Ultrastructural analyses of deciduous teeth affected by hypocalcified amelogenesis imperfecta from a family with a novel y458x fam83h nonsense mutation. *Cells, tissues, organs*, 191(3):235–239, 2010.

[65] W. El-Sayed, R. C. Shore, D. A. Parry, C. F. Inglehearn, and A. J. Mighell. Hypomaturation amelogenesis imperfecta due to wdr72 mutations: a novel mutation and ultrastructural analyses of deciduous teeth. *Cells, tissues, organs*, 194(1):60–66, 2011.

[66] J. C. Elliott, D. W. Holcomb, and R. A. Young. Infrared determination of the degree of substitution of hydroxyl by carbonate ions in human dental enamel. *Calcified tissue international*, 37(4):372–375, 1985.

[67] A. G. Fincham, Y. Hu, E. C. Lau, H. C. Slavkin, and M. L. Snead. Amelogenin post-secretory processing during biomineralization in the postnatal mouse molar tooth. *Archives of Oral Biology*, 36(4):305–317, 1991.

[68] A. G. Fincham, J. Moradian-Oldak, T. G. Diekwisch, D. M. Lyaruu, J. T. Wright, P. Bringas Jr, and H. C. Slavkin. Evidence for amelogenin "nanospheres" as functional components of secretory-stage enamel matrix. *Journal of structural biology*, 115(1):50–59, 1995.

[69] A. G. Fincham, J. Moradian-Oldak, and J. P. Simmer. The structural biology of the developing dental enamel matrix. *Journal of structural biology*, 126(3):270–299, 1999.

[70] A. G. Fincham, J. Moradian-Oldak, J. P. Simmer, P. Sarte, E. C. Lau, T. Diekwisch, and H. C. Slavkin. Self-assembly of a recombinant amelogenin protein generates supramolecular structures. *Journal of structural biology*, 112(2):103–109, 1994.

[71] L. Fontana, Y. Chen, P. Prijatelj, T. Sakai, R. Fassler, L. Y. Sakai, and D. B. Rifkin. Fibronectin is required for integrin alphavbeta6-mediated activation of latent tgf-beta complexes containing ltbp-1. *FASEB journal* , 19(13):1798–1808, 2005.

[72] M. Fukae, T. Tanabe, T. Uchida, S. K. Lee, O. H. Ryu, C. Murakami, K. Wakida, J. P. Simmer, Y. Yamada, and J. D. Bartlett. Enamelysin (matrix metalloproteinase-20): localization in the developing tooth and effects of ph and calcium on amelogenin hydrolysis. *Journal of dental research*, 77(8):1580–1588, 1998.

[73] S. Fukumoto, T. Kiba, B. Hall, N. Iehara, T. Nakamura, G. Longenecker, P. H. Krebsbach, A. Nanci, A. B. Kulkarni, and Y. Yamada. Ameloblastin is a cell adhesion molecule required for maintaining the differentiation state of ameloblasts. *The Journal of cell biology*, 167(5):973–983, 2004.

[74] S. Fukumoto, A. Yamada, K. Nonaka, and Y. Yamada. Essential roles of ameloblastin in maintaining ameloblast differentiation and enamel formation. *Cells, tissues, organs*, 181(3-4):189–195, 2005.

[75] B. Ganss, R. H. Kim, and J. Sodek. Bone sialoprotein. *Critical reviews in oral biology and medicine*, 10(1):79–98, 1999.

[76] S. Gao, C. Alarcon, G. Sapkota, S. Rahman, P. Y. Chen, N. Goerner, M. J. Macias, H. Erdjument-Bromage, P. Tempst, and J. Massague. Ubiquitin ligase nedd4l targets activated smad2/3 to limit tgf-beta signaling. *Molecular cell*, 36(3):457–468, 2009.

[77] Y. Gao, D. Li, T. Han, Y. Sun, and J. Zhang. Tgf-beta1 and tgfbr1 are expressed in ameloblasts and promote mmp20 expression. *Anatomical record (Hoboken, N.J.: 2007)*, 292(6):885–890, 2009.

[78] R. C. Gentleman, V. J. Carey, D. M. Bates, B. Bolstad, M. Dettling, S. Dudoit, B. Ellis, L. Gautier, Y. Ge, J. Gentry, K. Hornik, T. Hothorn, W. Huber, S. Iacus, R. Irizarry, F. Leisch, C. Li, M. Maechler, A. J. Rossini, G. Sawitzki, C. Smith, G. Smyth, L. Tierney, J. Y. Yang, and J. Zhang. Bioconductor: open software development for computational biology and bioinformatics. *Genome biology*, 5(10):R80, 2004.

[79] S. Gestrelius, C. Andersson, D. Lidstrom, L. Hammarstrom, and M. Somerman. In vitro studies on periodontal ligament cells and enamel matrix derivative. *Journal of clinical periodontology*, 24(9 Pt 2):685–692, 1997.

[80] F. Ghannad, D. Nica, M. I. Fulle, D. Grenier, E. E. Putnins, S. Johnston, A. Eslami, L. Koivisto, G. Jiang, M. D. McKee, L. Hakkinen, and H. Larjava. Absence of alphavbeta6 integrin is linked to initiation and progression of periodontal disease. *The American journal of pathology*, 172(5):1271–1286, 2008.

[81] F. G. Giancotti. Integrin signaling: specificity and control of cell survival and cell cycle progression. *Current opinion in cell biology*, 9(5):691–700, 1997.

[82] F. G. Giancotti and E. Ruoslahti. Integrin signaling. *Science (New York, N.Y.)*, 285(5430):1028–1032, 1999.

[83] C. W. Gibson, E. Golub, W. D. Ding, H. Shimokawa, M. Young, J. Termine, and J. Rosenbloom. Identification of the leucine-rich amelogenin peptide (lrap) as the translation product of an alternatively spliced transcript. *Biochemical and biophysical research communications*, 174(3):1306–1312, 1991.

[84] C. W. Gibson, Z. A. Yuan, B. Hall, G. Longenecker, E. Chen, T. Thyagarajan, T. Sreenath, J. T. Wright, S. Decker, R. Piddington, G. Harrison, and A. B. Kulkarni. Amelogenin-deficient mice display an amelogenesis imperfecta phenotype. *The Journal of biological chemistry*, 276(34):31871–31875, 2001.

[85] C. W. Gibson, Z. A. Yuan, Y. Li, B. Daly, C. Suggs, M. A. Aragon, F. Alawi, A. B. Kulkarni, and J. T. Wright. Transgenic mice that express normal and mutated amelogenins. *Journal of dental research*, 86(4):331–335, 2007.

[86] K. Gokce, C. Canpolat, and E. Ozel. Restoring function and esthetics in a patient with amelogenesis imperfecta: a case report. *The journal of contemporary dental practice*, 8(4):95–101, 2007.

[87] M. Goldberg, D. Septier, O. Rapoport, R. V. Iozzo, M. F. Young, and L. G. Ameye. Targeted disruption of two small leucine-rich proteoglycans, biglycan and decorin, excerpts divergent effects on enamel and dentin formation. *Calcified tissue international*, 77(5):297–310, 2005.

[88] S. R. Greene, Z. A. Yuan, J. T. Wright, H. Amjad, W. R. Abrams, J. A. Buchanan, D. I. Trachtenberg, and C. W. Gibson. A new frameshift mutation encoding a truncated amelogenin leads to x-linked amelogenesis imperfecta. *Archives of Oral Biology*, 47(3):211–217, 2002.

[89] A. Gritli-Linde, M. Bei, R. Maas, X. M. Zhang, A. Linde, and A. P. McMahon. Shh signaling within the dental epithelium is necessary for cell proliferation, growth and polarization. *Development (Cambridge, England)*, 129(23):5323–5337, 2002.

[90] S. J. Gutierrez, M. Chaves, D. M. Torres, and I. Briceno. Identification of a novel mutation in the enamalin gene in a family with autosomal-dominant amelogenesis imperfecta. *Archives of Oral Biology*, 52(5):503–506, 2007.

[91] K. Haapasalmi, K. Zhang, M. Tonnesen, J. Olerud, D. Sheppard, T. Salo, R. Kramer, R. A. Clark, V. J. Uitto, and H. Larjava. Keratinocytes in human wounds express alpha v beta 6 integrin. *The Journal of investigative dermatology*, 106(1):42–48, 1996.

[92] H. R. Haase and P. M. Bartold. Enamel matrix derivative induces matrix synthesis by cultured human periodontal fibroblast cells. *Journal of periodontology*, 72(3):341–348, 2001.

[93] K. Hahm, M. E. Lukashev, Y. Luo, W. J. Yang, B. M. Dolinski, P. H. Weinreb, K. J. Simon, L. Chun Wang, D. R. Leone, R. R. Lobb, D. J. McCrann, N. E. Allaire, G. S. Horan, A. Fogo, R. Kalluri, C. F. Shield 3rd, D. Sheppard, H. A. Gardner, and S. M. Violette. Alphav beta6 integrin regulates renal fibrosis and inflammation in alport mouse. *The American journal of pathology*, 170(1):110–125, 2007.

[94] L. Hakkinen, H. C. Hildebrand, A. Berndt, H. Kosmehl, and H. Larjava. Immunolocalization of tenascin-c, alpha9 integrin subunit, and alphavbeta6 integrin during wound healing in human oral mucosa. *The journal of histochemistry and cytochemistry*, 48(7):985–998, 2000.

[95] L. Hakkinen, L. Koivisto, H. Gardner, U. Saarialho-Kere, J. M. Carroll, M. Lakso, H. Rauvala, M. Laato, J. Heino, and H. Larjava. Increased expression of beta6-integrin in skin leads to spontaneous development of chronic wounds. *The American journal of pathology*, 164(1):229–242, 2004.

[96] L. Hakkinen, L. Koivisto, and H. Larjava. An improved method for culture of epidermal keratinocytes from newborn mouse skin. *Methods in cell science* , 23(4):189–196, 2001.

[97] S. Hamidi, T. Salo, T. Kainulainen, J. Epstein, K. Lerner, and H. Larjava. Expression of alpha(v)beta6 integrin in oral leukoplakia. *British journal of cancer*, 82(8):1433–1440, 2000.

[98] J. Hao, K. Narayanan, T. Muni, A. Ramachandran, and A. George. Dentin matrix protein 4, a novel secretory calcium-binding protein that modulates odontoblast differentiation. *The Journal of biological chemistry*, 282(21):15357–15365, 2007.

[99] H. Harada, T. Toyono, K. Toyoshima, M. Yamasaki, N. Itoh, S. Kato, K. Sekine, and H. Ohuchi. Fgf10 maintains stem cell compartment in developing mouse incisors. *Development (Cambridge, England)*, 129(6):1533–1541, 2002.

[100] D. Harmey, L. Hessle, S. Narisawa, K. A. Johnson, R. Terkeltaub, and J. L. Millan. Concerted regulation of inorganic pyrophosphate and osteopontin by akp2, enpp1, and ank: an integrated model of the pathogenesis of mineralization disorders. *The American journal of pathology*, 164(4):1199–1209, 2004.

[101] N. L. Harris, K. R. Rattray, C. E. Tye, T. M. Underhill, M. J. Somerman, J. A. D'Errico, A. F. Chambers, G. K. Hunter, and H. A. Goldberg. Functional analysis of bone sialoprotein: identification of the hydroxyapatite-nucleating and cell-binding domains by recombinant peptide expression and site-directed mutagenesis. *Bone*, 27(6):795–802, 2000.

[102] P. S. Hart, M. J. Aldred, P. J. Crawford, N. J. Wright, T. C. Hart, and J. T. Wright. Amelogenesis imperfecta phenotype-genotype correlations with two amelogenin gene mutations. *Archives of Oral Biology*, 47(4):261–265, 2002.

[103] P. S. Hart, S. Becerik, D. Cogulu, G. Emingil, D. Ozdemir-Ozenen, S. T. Han, P. P. Sulima, E. Firatli, and T. C. Hart. Novel fam83h mutations in turkish families with autosomal dominant hypocalcified amelogenesis imperfecta. *Clinical genetics*, 75(4):401–404, 2009.

[104] P. S. Hart, T. C. Hart, M. D. Michalec, O. H. Ryu, D. Simmons, S. Hong, and J. T. Wright. Mutation in kallikrein 4 causes autosomal recessive hypomaturation amelogenesis imperfecta. *Journal of medical genetics*, 41(7):545–549, 2004.

[105] P. S. Hart, M. Michalec, W. Seow, T. Hart, and J. Wright. Identification of a novel enamelin mutation (g.8344delg) in a family with amelogenesis imperfecta. *Arch.Oral Biol.*, 48:589–591, 2003.

[106] P. S. Hart, J. T. Wright, M. Savage, G. Kang, J. T. Bensen, M. C. Gorry, and T. C. Hart. Exclusion of candidate genes in two families with autosomal dominant hypocalcified amelogenesis imperfecta. *European journal of oral sciences*, 111(4):326–331, 2003.

[107] S. Hart, T. Hart, C. Gibson, and J. T. Wright. Mutational analysis of x-linked amelogenesis imperfecta in multiple families. *Archives of Oral Biology*, 45(1):79–86, 2000.

[108] T. C. Hart, P. S. Hart, M. C. Gorry, M. D. Michalec, O. H. Ryu, C. Uygur, D. Ozdemir, S. Firatli, G. Aren, and E. Firatli. Novel enam mutation responsible for autosomal recessive amelogenesis imperfecta and localised enamel defects. *Journal of medical genetics*, 40(12):900–906, 2003.

[109] N. Haruyama, T. Thyagarajan, Z. Skobe, J. T. Wright, D. Septier, T. L. Sreenath, M. Goldberg, and A. B. Kulkarni. Overexpression

of transforming growth factor-beta1 in teeth results in detachment of ameloblasts and enamel defects. *European journal of oral sciences*, 114 Suppl 1:30–4; discussion 39–41, 379, 2006.

[110] M. N. Helder, H. Karg, T. J. Bervoets, S. Vukicevic, E. H. Burger, R. N. D'Souza, J. H. Woltgens, G. Karsenty, and A. L. Bronckers. Bone morphogenetic protein-7 (osteogenic protein-1, op-1) and tooth development. *Journal of dental research*, 77(4):545–554, 1998.

[111] R. Heymann, S. Kallenbach, S. Alonso, P. Carroll, and T. A. Mitsiadis. Dynamic expression patterns of the new protocadherin families cnrs and pcdh-gamma during mouse odontogenesis: comparison with reelin expression. *Mechanisms of development*, 106(1-2):181–184, 2001.

[112] A. M. Hoang, T. W. Oates, and D. L. Cochran. In vitro wound healing responses to enamel matrix derivative. *Journal of periodontology*, 71(8):1270–1277, 2000.

[113] G. S. Horan, S. Wood, V. Ona, D. J. Li, M. E. Lukashev, P. H. Weinreb, K. J. Simon, K. Hahm, N. E. Allaire, N. J. Rinaldi, J. Goyal, C. A. Feghali-Bostwick, E. L. Matteson, C. O'Hara, R. Lafyatis, G. S. Davis, X. Huang, D. Sheppard, and S. M. Violette. Partial inhibition of integrin alpha(v)beta6 prevents pulmonary fibrosis without exacerbating inflammation. *American journal of respiratory and critical care medicine*, 177(1):56–65, 2008.

[114] C. C. Hu, M. Fukae, T. Uchida, Q. Qian, C. H. Zhang, O. H. Ryu, T. Tanabe, Y. Yamakoshi, C. Murakami, N. Dohi, M. Shimizu, and J. P. Simmer. Cloning and characterization of porcine enamelin mrnas. *Journal of dental research*, 76(11):1720–1729, 1997.

[115] C. C. Hu, M. Fukae, T. Uchida, Q. Qian, C. H. Zhang, O. H. Ryu, T. Tanabe, Y. Yamakoshi, C. Murakami, N. Dohi, M. Shimizu, and J. P. Simmer. Sheathlin: cloning, cdna/polypeptide sequences, and immunolocalization of porcine enamel sheath proteins. *Journal of dental research*, 76(2):648–657, 1997.

[116] C. C. Hu, T. C. Hart, B. R. Dupont, J. J. Chen, X. Sun, Q. Qian, C. H. Zhang, H. Jiang, V. L. Mattern, J. T. Wright, and J. P. Simmer. Cloning human enamelin cdna, chromosomal localization, and analysis of expression during tooth development. *Journal of dental research*, 79(4):912–919, 2000.

[117] J. C. Hu, H. C. Chan, S. G. Simmer, F. Seymen, A. S. Richardson, Y. Hu, R. N. Milkovich, N. M. Estrella, M. Yildirim, M. Bayram, C. F. Chen, and J. P. Simmer. Amelogenesis imperfecta in two families with defined amelx deletions in arhgap6. *PloS one*, 7(12):e52052, 2012.

[118] J. C. Hu, Y. H. Chun, T. Al Hazzazzi, and J. P. Simmer. Enamel formation and amelogenesis imperfecta. *Cells, tissues, organs*, 186(1):78–85, 2007.

[119] J. C. Hu, Y. Hu, C. E. Smith, M. D. McKee, J. T. Wright, Y. Yamakoshi, P. Papagerakis, G. K. Hunter, J. Q. Feng, F. Yamakoshi, and J. P. Simmer. Enamel defects and ameloblast-specific expression in enam knock-out/lacz knock-in mice. *The Journal of biological chemistry*, 283(16):10858–10871, 2008.

[120] J. C. Hu, X. Sun, C. Zhang, S. Liu, J. D. Bartlett, and J. P. Simmer. Enamelysin and kallikrein-4 mrna expression in developing mouse molars. *European journal of oral sciences*, 110(4):307–315, 2002.

[121] J. C. Hu and Y. Yamakoshi. Enamelin and autosomal-dominant amelogenesis imperfecta. *Critical reviews in oral biology and medicine*, 14(6):387–398, 2003.

[122] J. C. Hu, C. Zhang, X. Sun, Y. Yang, X. Cao, O. Ryu, and J. P. Simmer. Characterization of the mouse and human prss17 genes, their relationship to other serine proteases, and the expression of prss17 in developing mouse incisors. *Gene*, 251(1):1–8, 2000.

[123] J. C. Hu, C. H. Zhang, Y. Yang, C. Karrman-Mardh, K. Forsman-Semb, and J. P. Simmer. Cloning and characterization of the mouse and human enamelin genes. *Journal of dental research*, 80(3):898–902, 2001.

[124] X. Huang, J. Wu, S. Spong, and D. Sheppard. The integrin alphavbeta6 is critical for keratinocyte migration on both its known ligand, fibronectin, and on vitronectin. *Journal of cell science*, 111 (Pt 15)(Pt 15):2189–2195, 1998.

[125] X. Huang, J. Wu, W. Zhu, R. Pytela, and D. Sheppard. Expression of the human integrin beta6 subunit in alveolar type ii cells and bronchiolar epithelial cells reverses lung inflammation in beta6 knockout mice. *American journal of respiratory cell and molecular biology*, 19(4):636–642, 1998.

[126] X. Z. Huang, J. F. Wu, D. Cass, D. J. Erle, D. Corry, S. G. Young, R. V. Farese Jr, and D. Sheppard. Inactivation of the integrin beta 6 subunit gene reveals a role of epithelial integrins in regulating inflammation in the lung and skin. *The Journal of cell biology*, 133(4):921–928, 1996.

[127] R. O. Hynes. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell*, 69(1):11–25, 1992.

[128] R. O. Hynes. Integrins: bidirectional, allosteric signaling machines. *Cell*, 110(6):673–687, 2002.

[129] R. O. Hynes. The emergence of integrins: a personal and historical perspective. *Matrix biology : journal of the International Society for Matrix Biology*, 23(6):333–340, 2004.

[130] M. Hyytiainen, C. Penttinen, and J. Keski-Oja. Latent tgf-beta binding proteins: extracellular matrix association and roles in tgf-beta activation. *Critical reviews in clinical laboratory sciences*, 41(3):233–264, 2004.

[131] H. O. Ishikawa, A. Xu, E. Ogura, G. Manning, and K. D. Irvine. The raine syndrome protein fam20c is a golgi kinase that phosphorylates bio-mineralization proteins. *PloS one*, 7(8):e42988, 2012.

[132] Y. Ito, P. Sarkar, Q. Mi, N. Wu, P. Bringas Jr, Y. Liu, S. Reddy, R. Maxson, C. Deng, and Y. Chai. Overexpression of smad2 reveals its concerted action with smad4 in regulating tgf-beta-mediated epidermal homeostasis. *Developmental biology*, 236(1):181–194, 2001.

[133] K. Iwasaki, E. Bajenova, E. Somogyi-Ganss, M. Miller, V. Nguyen, H. Nourkeyhani, Y. Gao, M. Wendel, and B. Ganss. Amelotin–a novel secreted, ameloblast-specific protein. *Journal of dental research*, 84(12):1127–1132, 2005.

[134] T. Jackson, D. Sheppard, M. Denyer, W. Blakemore, and A. M. King. The epithelial integrin alphavbeta6 is a receptor for foot-and-mouth disease virus. *Journal of virology*, 74(11):4949–4956, 2000.

[135] S. C. Janga and N. Mittal. Construction, structure and dynamics of post-transcriptional regulatory network directed by rna-binding proteins. *Advances in Experimental Medicine and Biology*, 722:103–117, 2011.

[136] J. Jernvall and I. Thesleff. Reiterative signaling and patterning during mammalian tooth morphogenesis. *Mechanisms of development*, 92(1):19–29, 2000.

[137] M. F. Jobling, J. D. Mott, M. T. Finnegan, V. Jurukovski, A. C. Erickson, P. J. Walian, S. E. Taylor, S. Ledbetter, C. M. Lawrence, D. B. Rifkin, and M. H. Barcellos-Hoff. Isoform-specific activation of latent transforming growth factor beta (ltgf-beta) by reactive oxygen species. *Radiation research*, 166(6):839–848, 2006.

[138] K. Josephsen and O. Fejerskov. Ameloblast modulation in the maturation zone of the rat incisor enamel organ. a light and electron microscopic study. *Journal of anatomy*, 124(Pt 1):45–70, 1977.

[139] J. Jovanovic, J. Takagi, L. Choulier, N. G. Abrescia, D. I. Stuart, P. A. van der Merwe, H. J. Mardon, and P. A. Handford. alphavbeta6 is a novel receptor for human fibrillin-1. comparative studies of molecular determinants underlying integrin-rgd affinity and specificity. *The Journal of biological chemistry*, 282(9):6743–6751, 2007.

[140] M. Jussila and I. Thesleff. Signaling networks regulating tooth organogenesis and regeneration, and the specification of dental mesenchymal and epithelial cell lineages. *Cold Spring Harbor perspectives in biology*, 4(4):a008425, 2012.

[141] E. Kallenbach. The fine structure of tomes' process of rat incisor ameloblasts and its relationship to the elaboration of enamel. *Tissue & cell*, 5(3):501–524, 1973.

[142] H. Y. Kang, F. Seymen, S. K. Lee, M. Yildirim, E. B. Tuna, A. Patir, K. E. Lee, and J. W. Kim. Candidate gene strategy reveals enam mutations. *Journal of dental research*, 88(3):266–269, 2009.

[143] J. S. Kang, C. Liu, and R. Derynck. New regulatory mechanisms of tgf-beta receptor function. *Trends in cell biology*, 19(8):385–394, 2009.

[144] T. Kawase, K. Okuda, H. Yoshie, and D. M. Burns. Cytostatic action of enamel matrix derivative (emdogain) on human oral squamous cell carcinoma-derived scc25 epithelial cells. *Journal of periodontal research*, 35(5):291–300, 2000.

[145] M. Kida, T. Ariga, T. Shirakawa, H. Oguchi, and Y. Sakiyama. Autosomal-dominant hypoplastic form of amelogenesis imperfecta

caused by an enamelin gene mutation at the exon-intron boundary. *Journal of dental research*, 81(11):738–742, 2002.

[146] M. Kida, Y. Sakiyama, A. Matsuda, S. Takabayashi, H. Ochi, H. Sekiguchi, S. Minamitake, and T. Ariga. A novel missense mutation (p.p52r) in amelogenin gene causing x-linked amelogenesis imperfecta. *Journal of dental research*, 86(1):69–72, 2007.

[147] J. W. Kim, S. K. Lee, Z. H. Lee, J. C. Park, K. E. Lee, M. H. Lee, J. T. Park, B. M. Seo, J. C. Hu, and J. P. Simmer. Fam83h mutations in families with autosomal-dominant hypocalcified amelogenesis imperfecta. *American Journal of Human Genetics*, 82(2):489–494, 2008.

[148] J. W. Kim, F. Seymen, B. P. Lin, B. Kiziltan, K. Gencay, J. P. Simmer, and J. C. Hu. Enam mutations in autosomal-dominant amelogenesis imperfecta. *Journal of dental research*, 84(3):278–282, 2005.

[149] J. W. Kim, J. P. Simmer, T. C. Hart, P. S. Hart, M. D. Ramaswami, J. D. Bartlett, and J. C. Hu. Mmp-20 mutation in autosomal recessive pigmented hypomaturation amelogenesis imperfecta. *Journal of medical genetics*, 42(3):271–275, 2005.

[150] J. W. Kim, J. P. Simmer, Y. Y. Hu, B. P. Lin, C. Boyd, J. T. Wright, C. J. Yamada, S. K. Rayes, R. J. Feigal, and J. C. Hu. Amelogenin p.m1t and p.w4s mutations underlying hypoplastic x-linked amelogenesis imperfecta. *Journal of dental research*, 83(5):378–383, 2004.

[151] J. W. Kim, J. P. Simmer, B. P. Lin, F. Seymen, J. D. Bartlett, and J. C. Hu. Mutational analysis of candidate genes in 24 amelogenesis imperfecta families. *European journal of oral sciences*, 114 Suppl 1:3–12; discussion 39–41, 379, 2006.

[152] S. A. Kindelan, A. H. Brook, L. Gangemi, N. Lench, F. S. Wong, J. Fearne, Z. Jackson, G. Foster, and B. M. Stringer. Detection of a novel mutation in x-linked amelogenesis imperfecta. *Journal of dental research*, 79(12):1978–1982, 2000.

[153] B. Klopcic, T. Maass, E. Meyer, H. A. Lehr, D. Metzger, P. Chambon, A. Mann, and M. Blessing. Tgf-beta superfamily signaling is essential for tooth and hair morphogenesis and differentiation. *European journal of cell biology*, 86(11-12):781–799, 2007.

[154] T. Koike, T. Izumikawa, J. Tamura, and H. Kitagawa. Fam20b is a kinase that phosphorylates xylose in the glycosaminoglycan-protein linkage region. *The Biochemical journal*, 421(2):157–162, 2009.

[155] S. Kojima, K. Nara, and D. B. Rifkin. Requirement for transglutaminase in the activation of latent transforming growth factor-beta in bovine endothelial cells. *The Journal of cell biology*, 121(2):439–448, 1993.

[156] L. L. Koth, B. Alex, S. Hawgood, M. A. Nead, D. Sheppard, D. J. Erle, and D. G. Morris. Integrin beta6 mediates phospholipid and collectin homeostasis by activation of latent tgf-beta1. *American journal of respiratory cell and molecular biology*, 37(6):651–659, 2007.

[157] P. H. Krebsbach, S. K. Lee, Y. Matsuki, C. A. Kozak, K. M. Yamada, and Y. Yamada. Full-length sequence, localization, and chromosomal mapping of ameloblastin. a novel tooth-specific gene. *The Journal of biological chemistry*, 271(8):4431–4435, 1996.

[158] Y. S. Kweon, K. E. Lee, J. Ko, J. C. Hu, J. P. Simmer, and J. W. Kim. Effects of fam83h overexpression on enamel and dentine formation. *Archives of Oral Biology*, 58(9):1148–1154, 2013.

[159] R. S. Lacruz, A. Nanci, I. Kurtz, J. T. Wright, and M. L. Paine. Regulation of ph during amelogenesis. *Calcified tissue international*, 86(2):91–103, 2010.

[160] M. Lagerstrom, N. Dahl, Y. Nakahori, Y. Nakagome, B. Backman, U. Landegren, and U. Pettersson. A deletion in the amelogenin gene (amg) causes x-linked amelogenesis imperfecta (aih1). *Genomics*, 10(4):971–975, 1991.

[161] M. Lagerstrom-fermer, M. Nilsson, B. Bckman, E. C. Salido, C. Shapiro, U. Petterson, and U. Landegren. Amelogenin signal peptide mutation: correlation mutations in the amelogenin gene (amgx) and manifestation of amelogenin imperfecta. *Genomics*, 26:159–162, 1995.

[162] H. Larjava, L. Koivisto, L. Hakkinen, and J. Heino. Epithelial integrins with special reference to oral epithelia. *Journal of dental research*, 90(12):1367–1376, 2011.

[163] H. Larjava, J. Peltonen, S. K. Akiyama, S. S. Yamada, H. R. Gralnick, J. Uitto, and K. M. Yamada. Novel function for beta 1 integrins in keratinocyte cell-cell interactions. *The Journal of cell biology*, 110(3):803–815, 1990.

[164] H. Larjava, T. Salo, K. Haapasalmi, R. H. Kramer, and J. Heino. Expression of integrins and basement membrane components by wound keratinocytes. *The Journal of clinical investigation*, 92(3):1425–1435, 1993.

[165] E. C. Lau, T. K. Mohandas, L. J. Shapiro, H. C. Slavkin, and M. L. Snead. Human and mouse amelogenin gene loci are on the sex chromosomes. *Genomics*, 4(2):162–168, 1989.

[166] D. A. Lawrence, R. Pircher, C. Kryceve-Martinerie, and F. Jullien. Normal embryo fibroblasts release transforming growth factors in a latent form. *Journal of Cellular Physiology*, 121:184–188, 1984.

[167] K. E. Lee, S. K. Lee, S. E. Jung, S. J. Song, S. H. Cho, Z. H. Lee, and J. W. Kim. A novel mutation in the amelx gene and multiple crown resorptions. *European journal of oral sciences*, 119 Suppl 1:324–328, 2011.

[168] M. J. Lee, S. K. Lee, K. E. Lee, H. Y. Kang, H. S. Jung, and J. W. Kim. Expression patterns of the fam83h gene during murine tooth development. *Archives of Oral Biology*, 54(9):846–850, 2009.

[169] S. K. Lee, J. C. Hu, J. D. Bartlett, K. E. Lee, B. P. Lin, J. P. Simmer, and J. W. Kim. Mutational spectrum of fam83h: the c-terminal portion is required for tooth enamel calcification. *Human mutation*, 29(8):E95–9, 2008.

[170] M. L. LeFevre and R. S. Manly. Moisture, inorganic and organic contents of enamel and dentin from carious teeth. *Journal of American Dental Association*, 24:233–242, 1932.

[171] N. J. Lench, A. H. Brook, and G. B. Winter. Sscp detection of a nonsense mutation in exon 5 of the amelogenin gene (amgx) causing x-linked amelogenesis imperfecta (aih1). *Human molecular genetics*, 3(5):827–828, 1994.

[172] N. J. Lench and G. B. Winter. Characterisation of molecular defects in x-linked amelogenesis imperfecta (aih1). *Human mutation*, 5(3):251–259, 1995.

[173] M. O. Li, S. Sanjabi, and R. A. Flavell. Transforming growth factor-β controls development, homeostasis, and tolerance of t cells by regulatory t cell-dependent and -independent mechanisms. *Immunity*, 25:455–471, 2006.

[174] H. Limeback and A. Simic. Biochemical characterization of stable high molecular-weight aggregates of amelogenins formed during porcine enamel development. *Archives of Oral Biology*, 35(6):459–468, 1990.

[175] E. Llano, A. M. Pendas, V. Knauper, T. Sorsa, T. Salo, E. Salido, G. Murphy, J. P. Simmer, J. D. Bartlett, and C. Lopez-Otin. Identification and structural and functional characterization of human enamelysin (mmp-20). *Biochemistry*, 36(49):15101–15108, 1997.

[176] B. L. Loeys, J. Chen, E. R. Neptune, D. P. Judge, M. Podowski, T. Holm, J. Meyers, C. C. Leitch, N. Katsanis, N. Sharifi, F. L. Xu, L. A. Myers, P. J. Spevak, D. E. Cameron, J. De Backer, J. Hellemans, Y. Chen, E. C. Davis, C. L. Webb, W. Kress, P. Coucke, D. B. Rifkin, A. M. De Paepe, and H. C. Dietz. A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in tgfbr1 or tgfbr2. *Nature genetics*, 37:275–281, 2005.

[177] Y. Lu, P. Papagerakis, Y. Yamakoshi, J. C. Hu, J. D. Bartlett, and J. P. Simmer. Functions of klk4 and mmp-20 in dental enamel formation. *Biological chemistry*, 389(6):695–700, 2008.

[178] D. Ma, R. Zhang, Y. Sun, H. F. Rios, N. Haruyama, X. Han, A. B. Kulkarni, C. Qin, and J. Q. Feng. A novel role of periostin in postnatal tooth formation and mineralization. *The Journal of biological chemistry*, 286(6):4302–4309, 2011.

[179] L. J. Ma, H. Yang, A. Gaspert, G. Carlesso, M. M. Barty, J. M. Davidson, D. Sheppard, and A. B. Fogo. Transforming growth factor-beta-dependent and -independent pathways of induction of tubulointerstitial fibrosis in beta6(-/-) mice. *The American journal of pathology*, 163(4):1261–1273, 2003.

[180] M. MacDougall, B. R. DuPont, D. Simmons, B. Reus, P. Krebsbach, C. Karrman, G. Holmgren, R. J. Leach, and K. Forsman. Ameloblastin gene (ambn) maps within the critical region for autosomal dominant amelogenesis imperfecta at chromosome 4q21. *Genomics*, 41(1):115–118, 1997.

[181] C. K. Mardh, B. Backman, G. Holmgren, J. C. Hu, J. P. Simmer, and K. Forsman-Semb. A nonsense mutation in the enamelin gene causes local hypoplastic autosomal dominant amelogenesis imperfecta (aih2). *Human molecular genetics*, 11(9):1069–1074, 2002.

[182] J. Massague, S. W. Blain, and R. S. Lo. Tgfbeta signaling in growth control, cancer, and heritable disorders. *Cell*, 103(2):295–309, 2000.

[183] H. Masuya, K. Shimizu, H. Sezutsu, Y. Sakuraba, J. Nagano, A. Shimizu, N. Fujimoto, A. Kawai, I. Miura, H. Kaneda, K. Kobayashi, J. Ishijima, T. Maeda, Y. Gondo, T. Noda, S. Wakana, and T. Shiroishi. Enamelin (enam) is essential for amelogenesis: Enu-induced mouse mutants as models for different clinical subtypes of human amelogenesis imperfecta (ai). *Human molecular genetics*, 14(5):575–583, 2005.

[184] E. B. McGowan, E. Becker, and T. C. Detwiler. Inhibition of calpain in intact platelets by the thiol protease inhibitor e-64d. *Biochemical and biophysical research communications*, 158(2):432–435, 1989.

[185] M. D. McKee and A. Nanci. Osteopontin and the bone remodeling sequence. colloidal-gold immunocytochemistry of an interfacial extracellular matrix protein. *Annals of the New York Academy of Sciences*, 760:177–189, 1995.

[186] M. D. McKee, S. Zalzal, and A. Nanci. Extracellular matrix in tooth cementum and mantle dentin: localization of osteopontin and other noncollagenous proteins, plasma proteins, and glycoconjugates by electron microscopy. *The Anatomical Record*, 245(2):293–312, 1996.

[187] F. Michon, M. Tummers, M. Kyyronen, M. J. Frilander, and I. Thesleff. Tooth morphogenesis and ameloblast differentiation are regulated by micro-rnas. *Developmental biology*, 340(2):355–368, 2010.

[188] S. E. Millar, E. Koyama, S. T. Reddy, T. Andl, T. Gaddapara, R. Piddington, and C. W. Gibson. Over- and ectopic expression of wnt3 causes progressive loss of ameloblasts in postnatal mouse incisor teeth. *Connective tissue research*, 44 Suppl 1:124–129, 2003.

[189] L. C. Miller, W. Blakemore, D. Sheppard, A. Atakilit, A. M. King, and T. Jackson. Role of the cytoplasmic domain of the beta-subunit of integrin alpha(v)beta6 in infection by foot-and-mouth disease virus. *Journal of virology*, 75(9):4158–4164, 2001.

[190] K. Miyazono, A. Olofsson, P. Colosetti, and C. H. Heldin. A role of the latent tgf-beta 1-binding protein in the assembly and secretion of tgf-beta 1. *The EMBO journal*, 10(5):1091–1101, 1991.

[191] P. Moffatt, C. E. Smith, R. Sooknanan, R. St-Arnaud, and A. Nanci. Identification of secreted and membrane proteins in the rat incisor enamel organ using a signal-trap screening approach. *European journal of oral sciences*, 114 Suppl 1:139–46; discussion 164–5, 380–1, 2006.

[192] P. Moffatt, C. E. Smith, R. St-Arnaud, D. Simmons, J. T. Wright, and A. Nanci. Cloning of rat amelotin and localization of the protein to the basal lamina of maturation stage ameloblasts and junctional epithelium. *The Biochemical journal*, 399(1):37–46, 2006.

[193] L. Mohazab, L. Koivisto, G. Jiang, L. Kytömäki, M. Haapasalo, G. R. Owen, C. Wiebe, Y. Xie, K. Heikinheimo, T. Yoshida, et al. Critical role for αvβ6 integrin in enamel biomineralization. *Journal of cell science*, 126(3):732–744, 2013.

[194] H. Morishita and T. Yagi. Protocadherin family: diversity, structure, and function. *Current opinion in cell biology*, 19(5):584–592, 2007.

[195] A. Moustakas and C. H. Heldin. Dynamic control of tgf-beta signaling and its links to the cytoskeleton. *FEBS letters*, 582(14):2051–2065, 2008.

[196] J. S. Munger, X. Huang, H. Kawakatsu, M. J. Griffiths, S. L. Dalton, J. Wu, J. F. Pittet, N. Kaminski, C. Garat, M. A. Matthay, D. B. Rifkin, and D. Sheppard. The integrin alpha v beta 6 binds and activates latent tgf beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell*, 96(3):319–328, 1999.

[197] T. Mustonen, M. Ilmonen, M. Pummila, A. T. Kangas, J. Laurikkala, R. Jaatinen, J. Pispa, O. Gaide, P. Schneider, I. Thesleff, and M. L. Mikkola. Ectodysplasin a1 promotes placodal cell fate during early morphogenesis of ectodermal appendages. *Development (Cambridge, England)*, 131(20):4907–4919, 2004.

[198] T. Nagano, A. Kakegawa, Y. Yamakoshi, S. Tsuchiya, J. C. Hu, K. Gomi, T. Arai, J. D. Bartlett, and J. P. Simmer. Mmp-20 and klk4 cleavage site preferences for amelogenin sequences. *Journal of dental research*, 88(9):823–828, 2009.

[199] T. Nagano, S. Oida, H. Ando, K. Gomi, T. Arai, and M. Fukae. Relative levels of mrna encoding enamel proteins in enamel organ epithelia and odontoblasts. *Journal of dental research*, 82(12):982–986, 2003.

[200] Y. Nakahori, O. Takenaka, and Y. Nakagome. A human x-y homologous region encodes "amelogenin". *Genomics*, 9(2):264–269, 1991.

[201] T. Nakamura, S. de Vega, S. Fukumoto, L. Jimenez, F. Unda, and Y. Yamada. Transcription factor epiprofin is essential for tooth morphogenesis by regulating epithelial cell fate and tooth number. *The Journal of biological chemistry*, 283(8):4825–4833, 2008.

[202] D. Nalbant, H. Youn, S. I. Nalbant, S. Sharma, E. Cobos, E. G. Beale, Y. Du, and S. C. Williams. Fam20: an evolutionarily conserved family of secreted proteins expressed in hematopoietic cells. *BMC genomics*, 6:11, 2005.

[203] A. Nanci and C. E. Smith. *Matrix-mediated mineralization in enamel and the collagen-based hard tissues*, chapter 35, pages 217–224. Chemistry and Biology of Mineralized Tissues. Rosemont, 2000.

[204] N. Narani, G. R. Owen, L. Hakkinen, E. Putnins, and H. Larjava. Enamel matrix proteins bind to wound matrix proteins and regulate their cell-adhesive properties. *European journal of oral sciences*, 115(4):288–295, 2007.

[205] A. H. Nawfal, D. Sidney, and J-Y Sire. Mammalian enamelins: identification of conserved regions, evolution mode and made use of for validation of mutations leading to amelogenesis imperfecta. *European Cells and Materials Journal*, 14:67, 2007.

[206] J. Dos Santos Neves, R. M. Wazen, S. Kuroda, S. Francis Zalzal, P. Moffatt, and A. Nanci. Odontogenic ameloblast-associated and amelotin are novel basal lamina components. *Histochemistry and cell biology*, 137(3):329–338, 2012.

[207] F. K. Ng and L. B. Messer. Dental management of amelogenesis imperfecta patients: a primer on genotype-phenotype correlations. *Pediatric dentistry*, 31(1):20–30, 2009.

[208] S. L. Nishimura. Integrin-mediated transforming growth factor-beta activation, a potential therapeutic target in fibrogenic disorders. *The American journal of pathology*, 175(4):1362–1370, 2009.

[209] E. Ostergaard, M. Batbayli, M. Duno, K. Vilhelmsen, and T. Rosenberg. Mutations in pcdh21 cause autosomal recessive cone-rod dystrophy. *Journal of medical genetics*, 47(10):665–669, 2010.

[210] J. O'Sullivan, C. C. Bitu, S. B. Daly, J. E. Urquhart, M. J. Barron, S. S. Bhaskar, H. Martelli-Junior, P. E. dos Santos Neto, M. A. Mansilla, J. C. Murray, R. D. Coletta, G. C. Black, and M. J. Dixon. Whole-exome sequencing identifies fam20a mutations as a cause of amelogenesis imperfecta and gingival hyperplasia syndrome. *American Journal of Human Genetics*, 88(5):616–620, 13 2011.

[211] D. Ozdemir, P. S. Hart, E. Firatli, G. Aren, O. H. Ryu, and T. C. Hart. Phenotype of enam mutations is dosage-dependent. *Journal of dental research*, 84(11):1036–1041, 2005.

[212] D. Ozdemir, P. S. Hart, O. H. Ryu, S. J. Choi, M. Ozdemir-Karatas, E. Firatli, N. Piesco, and T. C. Hart. Mmp20 active-site mutation in hypomaturation amelogenesis imperfecta. *Journal of dental research*, 84(11):1031–1035, 2005.

[213] M. L. Paine, W. Luo, H. J. Wang, P. Bringas Jr, A. Y. Ngan, V. G. Miklus, D. H. Zhu, M. MacDougall, S. N. White, and M. L. Snead. Dentin sialoprotein and dentin phosphoprotein overexpression during amelogenesis. *The Journal of biological chemistry*, 280(36):31991–31998, 2005.

[214] M. L. Paine, D. H. Zhu, W. Luo, and M. L. Snead. Overexpression of trap in the enamel matrix does not alter the enamel structural hierarchy. *Cells, tissues, organs*, 176(1-3):7–16, 2004.

[215] P. Papagerakis, H. K. Lin, K. Y. Lee, Y. Hu, J. P. Simmer, J. D. Bartlett, and J. C. Hu. Premature stop codon in mmp20 causing amelogenesis imperfecta. *Journal of dental research*, 87(1):56–59, 2008.

[216] D. A. Parry, A. J. Mighell, W. El-Sayed, R. C. Shore, I. K. Jalili, H. Dollfus, A. Bloch-Zupan, R. Carlos, I. M. Carr, L. M. Downey, K. M. Blain, D. C. Mansfield, M. Shahrabi, M. Heidari, P. Aref, M. Abbasi, M. Michaelides, A. T. Moore, J. Kirkham, and C. F. Inglehearn. Mutations in cnnm4 cause jalili syndrome, consisting of autosomal-recessive cone-rod dystrophy and amelogenesis imperfecta. *American Journal of Human Genetics*, 84(2):266–273, 2009.

[217] E. Patsenker, Y. Popov, F. Stickel, A. Jonczyk, S. L. Goodman, and D. Schuppan. Inhibition of integrin alphavbeta6 on cholangiocytes blocks transforming growth factor-beta activation and retards biliary fibrosis progression. *Gastroenterology*, 135(2):660–670, 2008.

[218] A. Pavlic, M. Petelin, and T. Battelino. Phenotype and enamel ultrastructure characteristics in patients with enam gene mutations g.13185-13186insag and 8344delg. *Archives of Oral Biology*, 52(3):209–217, 2007.

[219] R. W. Pelton, B. Saxena, M. Jones, H. L. Moses, and L. I. Gold. Immunohistochemical localization of tgf beta 1, tgf beta 2, and tgf beta 3 in the mouse embryo: expression patterns suggest multiple roles during embryonic development. *The Journal of cell biology*, 115(4):1091–1105, 1991.

[220] J. J. Pindborg and J. P. Weinmann. Morphologic and functional correlations in the enamel organ of the rat incisor during amelogenesis. *Acta Anatomica*, 36(4):367–381, 1959.

[221] J. F. Pittet, M. J. Griffiths, T. Geiser, N. Kaminski, S. L. Dalton, X. Huang, L. A. Brown, P. J. Gotwals, V. E. Koteliansky, M. A. Matthay, and D. Sheppard. Tgf-β is a critical mediator of acute lung injury. *The Journal of clinical investigation*, 107:1537–1544, 2001.

[222] R. A. Poche, R. Sharma, M. D. Garcia, A. M. Wada, M. J. Nolte, R. S. Udan, J. H. Paik, R. A. DePinho, J. D. Bartlett, and M. E. Dickinson. Transcription factor foxo1 is essential for enamel biomineralization. *PloS one*, 7(1):e30357, 2012.

[223] B. Polok, P. Escher, A. Ambresin, E. Chouery, S. Bolay, I. Meunier, F. Nan, C. Hamel, F. L. Munier, B. Thilo, A. Megarbane, and D. F. Schorderet. Mutations in cnnm4 cause recessive cone-rod dystrophy with amelogenesis imperfecta. *American Journal of Human Genetics*, 84(2):259–265, 2009.

[224] S. Poulsen, H. Gjorup, D. Haubek, G. Haukali, H. Hintze, H. Lovschall, and M. Errboe. Amelogenesis imperfecta - a systematic literature review of associated dental and oro-facial abnormalities and their impact on patients. *Acta Odontologica Scandinavica*, 66(4):193–199, 2008.

[225] S. K. Prakash, R. Paylor, S. Jenna, N. Lache-Vane, D. L. Armstrong, B. Xu, M. A. Mancini, and H. Y. Zoghbi. Functional analysis of

arhgap6, a novel gtpase-activating protein for rhoa. *Human molecular genetics*, 9(4):477–488, 2000.

[226] J. A. Price, D. W. Bowden, J. T. Wright, M. J. Pettenati, and T. C. Hart. Identification of a mutation in dlx3 associated with tricho-dento-osseous (tdo) syndrome. *Human molecular genetics*, 7(3):563–569, 1998.

[227] A. L. Prieto, G. M. Edelman, and K. L. Crossin. Multiple integrins mediate cell attachment to cytotactin/tenascin. *Proceedings of the National Academy of Sciences of the United States of America*, 90(21):10154–10158, 1993.

[228] K. Puthawala, N. Hadjiangelis, S. C. Jacoby, E. Bayongan, Z. Zhao, Z. Yang, M. L. Devitt, G. S. Horan, P. H. Weinreb, M. E. Lukashev, S. M. Violette, K. S. Grant, C. Colarossi, S. C. Formenti, and J. S. Munger. Inhibition of integrin alpha(v)beta6, an activator of latent transforming growth factor-beta, prevents radiation-induced lung fibrosis. *American journal of respiratory and critical care medicine*, 177(1):82–90, 2008.

[229] R. Quinonez, R. Hoover, and J. T. Wright. Transitional anterior esthetic restorations for patients with enamel defects. *Pediatric dentistry*, 22(1):65–67, 2000.

[230] S. Rajagopal, K. Rajagopal, and R. J. Lefkowitz. Teaching old receptors new tricks: biasing seven-transmembrane receptors. *Nature reviews.Drug discovery*, 9(5):373–386, 2010.

[231] M. H. Rajpar, K. Harley, C. Laing, R. M. Davies, and M. J. Dixon. Mutation of the gene encoding the enamel-specific protein, enamelin, causes autosomal-dominant amelogenesis imperfecta. *Human molecular genetics*, 10(16):1673–1677, 2001.

[232] D. B. Ravassipour, P. S. Hart, T. C. Hart, A. V. Ritter, M. Yamauchi, C. Gibson, and J. T. Wright. Unique enamel phenotype associated with amelogenin gene (amelx) codon 41 point mutation. *Journal of dental research*, 79(7):1476–1481, 2000.

[233] E. J. Reith. The stages of amelogenesis as observed in molar teeth of young rats. *Journal of Ultrastructure Research*, 30:111–151, 1970.

[234] E. J. Reith and A. Boyde. Histochemical and electron probe analysis of secretory ameloblasts of developing rat molar teeth. *Histochemistry*, 55(1):17–26, 1978.

[235] H. Rios, S. V. Koushik, H. Wang, J. Wang, H. M. Zhou, A. Lindsley, R. Rogers, Z. Chen, M. Maeda, A. Kruzynska-Frejtag, J. Q. Feng, and S. J. Conway. Periostin null mice exhibit dwarfism, incisor enamel defects, and an early-onset periodontal disease-like phenotype. *Molecular and cellular biology*, 25(24):11131–11144, 2005.

[236] S. Risnes. Growth tracks in dental enamel. *Journal of human evolution*, 35(4-5):331–350, 1998.

[237] E. Ronnholm. The amelogenesis of human teeth as revealed by electron microscopy. i: The fine structure of the ameloblasts. *J.Ultrastruct.Res.*, 6:229–248, 1962.

[238] E. Ronnholm. The amelogenesis of human teeth as revealed by electron microscopy. ii. the development of the enamel crystallites. *Journal of ultrastructure research*, 6:249–303, 1962.

[239] I. Ruspita, K. Miyoshi, T. Muto, K. Abe, T. Horiguchi, and T. Noma. Sp6 downregulation of follistatin gene expression in ameloblasts. *The journal of medical investigation : JMI*, 55(1-2):87–98, 2008.

[240] M. C. Ryan, K. Lee, Y. Miyashita, and W. G. Carter. Targeted disruption of the lama3 gene in mice reveals abnormalities in survival and late stage differentiation of epithelial cells. *The Journal of cell biology*, 145(6):1309–1323, 1999.

[241] O. Ryu, J. C. Hu, Y. Yamakoshi, J. L. Villemain, X. Cao, C. Zhang, J. D. Bartlett, and J. P. Simmer. Porcine kallikrein-4 activation, glycosylation, activity, and expression in prokaryotic and eukaryotic hosts. *European journal of oral sciences*, 110(5):358–365, 2002.

[242] C. Sabatini and S. Guzman-Armstrong. A conservative treatment for amelogenesis imperfecta with direct resin composite restorations: a case report. *Journal of esthetic and restorative dentistry*, 21(3):161–9; discussion 170, 2009.

[243] M. K. Saha and S. G. Saha. Restoration of anterior teeth with direct composite veneers in amelogenesis imperfecta. *International Journal of Dental Clinics*, 3(2):99–100, 2011.

[244] J. Saharinen and J. Keski-Oja. Specific sequence motif of 8-cys repeats of tgf-beta binding proteins, ltbps, creates a hydrophobic interaction surface for binding of small latent tgf-beta. *Molecular biology of the cell*, 11(8):2691–2704, 2000.

[245] E. C. Salido, P. H. Yen, K. Koprivnikar, L. C. Yu, and L. J. Shapiro. The human enamel protein gene amelogenin is expressed from both the x and the y chromosomes. *American Journal of Human Genetics*, 50(2):303–316, 1992.

[246] K. Salmivirta, D. Gullberg, E. Hirsch, F. Altruda, and P. Ekblom. Integrin subunit expression associated with epithelial-mesenchymal interactions during murine tooth development. *Developmental dynamics* , 205(2):104–113, 1996.

[247] C. Sanchez-Quevedo, G. Ceballos, I. A. Rodriguez, J. M. Garcia, and M. Alaminos. Acid-etching effects in hypomineralized amelogenesis imperfecta. a microscopic and microanalytical study. *Medicina oral, patologia oral y cirugia bucal*, 11(1):E40–3, 2006.

[248] A. R. Espirito Santo, J. D. Bartlett, C. W. Gibson, Y. Li, A. B. Kulkarni, and S. R. Line. Amelogenin- and enamelysin (mmp-20)-deficient mice display altered birefringence in the secretory-stage enamel organic extracellular matrix. *Connective tissue research*, 48(1):39–45, 2007.

[249] A. P. Pires Dos Santos, C. M. Cabral, L. F. Moliterno, and B. H. Oliveira. Amelogenesis imperfecta: report of a successful transitional treatment in the mixed dentition. *Journal of dentistry for children (Chicago, Ill.)*, 75(2):201–206, 2008.

[250] S. Sapir and J. Shapira. Clinical solutions for developmental defects of enamel and dentin in children. *Pediatric dentistry*, 29(4):330–336, 2007.

[251] I. Satokata, L. Ma, H. Ohshima, M. Bei, I. Woo, K. Nishizawa, T. Maeda, Y. Takano, M. Uchiyama, S. Heaney, H. Peters, Z. Tang, R. Maxson, and R. Maas. Msx2 deficiency in mice causes pleiotropic defects in bone growth and ectodermal organ formation. *Nature genetics*, 24(4):391–395, 2000.

[252] H. E. Schroeder and M. A. Listgarten. Fine structure of the developing epithelial attachment of human teeth. *Monographs in developmental biology*, 2:1–134, 1971.

[253] H. Seedorf, M. Klaften, F. Eke, H. Fuchs, U. Seedorf, and M. Hrabe de Angelis. A mutation in the enamelin gene in a mouse model. *Journal of dental research*, 86(8):764–768, 2007.

[254] H. Seedorf, I. N. Springer, E. Grundner-Culemann, H. K. Albers, A. Reis, H. Fuchs, M. Hrabe de Angelis, and Y. Acil. Amelogenesis imperfecta in a new animal model–a mutation in chromosome 5 (human 4q21). *Journal of dental research*, 83(8):608–612, 2004.

[255] J. Seitsonen, P. Susi, O. Heikkila, R. S. Sinkovits, P. Laurinmaki, T. Hyypia, and S. J. Butcher. Interaction of alphavbeta3 and alphavbeta6 integrins with human parechovirus 1. *Journal of virology*, 84(17):8509–8519, 2010.

[256] H. Sekiguchi, S. Alaluusua, K. Minaguchi, and M. Yakushiji. A new mutation in the amelogenin gene causes x-linked amelogenesis imperfecta. *J.Dent.Res.*, 80:617, 2001.

[257] H. Sekiguchi, M. Kiyoshi, and M. Yakushiji. Dna diagnosis of x-linked amelogenesis imperfecta using pcr detection method of the human amelogenin gene. *Dent. Jpn.*, 37:109–112, 2001.

[258] W. K. Seow and A. Amaratunge. The effects of acid-etching on enamel from different clinical variants of amelogenesis imperfecta: an sem study. *Pediatric dentistry*, 20(1):37–42, 1998.

[259] J. L. Shapiro, X. Wen, C. T. Okamoto, H. J. Wang, S. P. Lyngstadaas, M. Goldberg, M. L. Snead, and M. L. Paine. Cellular uptake of amelogenin, and its localization to cd63, and lamp1-positive vesicles. *Cellular and molecular life sciences : CMLS*, 64(2):244–256, 2007.

[260] D. Sheppard, C. Rozzo, L. Starr, V. Quaranta, D. J. Erle, and R. Pytela. Complete amino acid sequence of a novel integrin beta subunit (beta 6) identified in epithelial cells using the polymerase chain reaction. *The Journal of biological chemistry*, 265(20):11502–11507, 15 1990.

[261] M. M. Shull, I. Ormsby, A. B. Kier, S. Pawlowski, R. J. Diebold, M. Yin, R. Allen, C. Sidman, G. Proetzel, and D. Calvin. Targeted

disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature*, 359(6397):693–699, 1992.

[262] J. P. Simmer and A. G. Fincham. Molecular mechanisms of dental enamel formation. *Crit.Rev.Oral Biol.Med.*, 6(2):84–108, 1995.

[263] J. P. Simmer and J. C. Hu. Dental enamel formation and its impact on clinical dentistry. *Journal of dental education*, 65(9):896–905, 2001.

[264] J. P. Simmer and J. C. Hu. Expression, structure, and function of enamel proteinases. *Connective tissue research*, 43(2-3):441–449, 2002.

[265] J. P. Simmer, Y. Hu, R. Lertlam, Y. Yamakoshi, and J. C. Hu. Hypomaturation enamel defects in klk4 knockout/lacz knockin mice. *The Journal of biological chemistry*, 284(28):19110–19121, 2009.

[266] J. P. Simmer, P. Papagerakis, C. E. Smith, D. C. Fisher, A. N. Rountrey, L. Zheng, and J. C. Hu. Regulation of dental enamel shape and hardness. *Journal of dental research*, 89(10):1024–1038, 2010.

[267] S. G. Simmer, N. M. Estrella, R. N. Milkovich, and J. C. Hu. Autosomal dominant amelogenesis imperfecta associated with enam frameshift mutation p.asn36ilefs56. *Clinical genetics*, 83(2):195–197, 2013.

[268] M. A. Simpson, R. Hsu, L. S. Keir, J. Hao, G. Sivapalan, L. M. Ernst, E. H. Zackai, L. I. Al-Gazali, G. Hulskamp, H. M. Kingston, T. E. Prescott, A. Ion, M. A. Patton, V. Murday, A. George, and A. H. Crosby. Mutations in fam20c are associated with lethal osteosclerotic bone dysplasia (raine syndrome), highlighting a crucial molecule in bone development. *American Journal of Human Genetics*, 81(5):906–912, 2007.

[269] C. E. Smith. Ameloblasts: secretory and resorptive functions. *Journal of dental research*, 58(Spec Issue B):695–707, 1979.

[270] C. E. Smith. Cellular and chemical events during enamel maturation. *Critical reviews in oral biology and medicine*, 9(2):128–161, 1998.

[271] C. E. Smith, D. L. Chong, J. D. Bartlett, and H. C. Margolis. Mineral acquisition rates in developing enamel on maxillary and mandibular incisors of rats and mice: implications to extracellular acid loading as apatite crystals mature. *Journal of bone and mineral research*, 20(2):240–249, 2005.

[272] C. E. Smith, M. Issid, H. C. Margolis, and E. C. Moreno. Developmental changes in the ph of enamel fluid and its effects on matrix-resident proteinases. *Advances in Dental Research*, 10:159–169, 1996.

[273] C. E. Smith and A. Nanci. A method for sampling the stages of amelogenesis on mandibular rat incisors using the molars as a reference for dissection. *The Anatomical Record*, 225(3):257–266, 1989.

[274] C. E. Smith, A. S. Richardson, Y. Hu, J. D. Bartlett, J. C. Hu, and J. P. Simmer. Effect of kallikrein 4 loss on enamel mineralization: comparison with mice lacking matrix metalloproteinase 20. *The Journal of biological chemistry*, 286(20):18149–18160, 2011.

[275] C. E. Smith, R. Wazen, Y. Hu, S. F. Zalzal, A. Nanci, J. P. Simmer, and J. C. Hu. Consequences for enamel development and mineralization resulting from loss of function of ameloblastin or enamelin. *European journal of oral sciences*, 117(5):485–497, 2009.

[276] M. L. Snead, M. Zeichner-David, T. Chandra, K. J. Robson, S. L. Woo, and H. C. Slavkin. Construction and identification of mouse amelogenin cdna clones. *Proceedings of the National Academy of Sciences of the United States of America*, 80(23):7254–7258, 1983.

[277] G. Stephanopoulos, M. E. Garefalaki, and K. Lyroudia. Genes and related proteins involved in amelogenesis imperfecta. *Journal of dental research*, 84(12):1117–1126, 2005.

[278] N. Suzuki, M. Ohyama, M. Maeno, K. Ito, and K. Otsuka. Attachment of human periodontal ligament cells to enamel matrix-derived protein is mediated via interaction between bsp-like molecules and integrin alpha(v)beta3. *Journal of periodontology*, 72(11):1520–1526, 2001.

[279] S. Suzuki and Y. Naitoh. Amino acid sequence of a novel integrin beta 4 subunit and primary expression of the mrna in epithelial cells. *The EMBO journal*, 9(3):757–763, 1990.

[280] J. Taipale, J. Saharinen, and J. Keski-Oja. Extracellular matrix-associated transforming growth factor-beta: role in cancer cell growth and invasion. *Advances in Cancer Research*, 75:87–134, 1998.

[281] Y. Takano. Cytochemical studies of ameloblasts and the surface layer of enamel of the rat incisor at the maturation stage. *Archivum histologicum Japonicum = Nihon soshikigaku kiroku*, 42(1):11–32, 1979.

[282] Y. Takano. Enamel mineralization and the role of ameloblasts in calcium transport. *Connective tissue research*, 33(1-3):127–137, 1995.

[283] R. N. Tamura, C. Rozzo, L. Starr, J. Chambers, L. F. Reichardt, H. M. Cooper, and V. Quaranta. Epithelial integrin $\alpha 6\beta 4$: complete primary structure of $\alpha 6$ variant forms of $\beta 4$. *The Journal of cell biology*, 111:1593–1604, 1990.

[284] T. Tanabe. Purification and characterization of proteolytic enzymes in porcine immature enamel. *Tsurumi shigaku.Tsurumi University dental journal*, 10(3):443–452, 1984.

[285] G. Tarone, E. Hirsch, M. Brancaccio, M. De Acetis, L. Barberis, F. Balzac, S. F. Retta, C. Botta, F. Altruda, and L. Silengo. Integrin function and regulation in development. *The International journal of developmental biology*, 44(6):725–731, 2000.

[286] R Development Core Team. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, 2008.

[287] I. Thesleff. Epithelial-mesenchymal signalling regulating tooth morphogenesis. *Journal of cell science*, 116(Pt 9):1647–1648, 2003.

[288] I. Thesleff. The genetic basis of tooth development and dental defects. *American journal of medical genetics.Part A*, 140(23):2530–2535, 2006.

[289] I. Thesleff and K. Hurmerinta. Tissue interactions in tooth development. *Differentiation*, 18:75–88, 1981.

[290] I. Thesleff, A. Vaahtokari, P. Kettunen, and T. Aberg. Epithelial-mesenchymal signaling during tooth development. *Connective tissue research*, 32:9–15, 1995.

[291] G. J. Thomas, M. L. Nystrom, and J. F. Marshall. Alphavbeta6 integrin in wound healing and cancer of the oral cavity. *Journal of oral pathology & medicine*, 35(1):1–10, 2006.

[292] M. Tummers and I. Thesleff. Root or crown: a developmental choice orchestrated by the differential regulation of the epithelial stem cell niche in the tooth of two rodent species. *Development (Cambridge, England)*, 130(6):1049–1057, 2003.

[293] M. Tummers and I. Thesleff. Observations on continuously growing roots of the sloth and the k14-eda transgenic mice indicate that epithelial stem cells can give rise to both the ameloblast and root epithelium cell lineage creating distinct tooth patterns. *Evolution & development*, 10(2):187–195, 2008.

[294] M. Tummers and I. Thesleff. The importance of signal pathway modulation in all aspects of tooth development. *Journal of experimental zoology.Part B, Molecular and developmental evolution*, 312B(4):309–319, 2009.

[295] T. Uchida, M. Fukae, T. Tanabe, Y. Yamakoshi, T. Satoda, C. Murakami, O. Takahashi, and M. Shimizu. Immunochemical and immunocytochemical study of a 15 kda non-amelogenin and related proteins in the porcine immature enamel: Proposal of a new group of enamel proteins sheath proteins. *Biomed Res*, 16:131–140, 1995.

[296] T. Uchida, C. Murakami, K. Wakida, N. Dohi, Y. Iwai, J. P. Simmer, M. Fukae, T. Satoda, and O. Takahashi. Sheath proteins: synthesis, secretion, degradation and fate in forming enamel. *European journal of oral sciences*, 106 Suppl 1:308–314, 1998.

[297] T. Uchida, T. Tanabe, M. Fukae, M. Shimizu, M. Yamada, K. Miake, and S. Kobayashi. Immunochemical and immunohistochemical studies, using antisera against porcine 25 kda amelogenin, 89 kda enamelin and the 13-17 kda nonamelogenins, on immature enamel of the pig and rat. *Histochemistry*, 96(2):129–138, 1991.

[298] M. T. van der Pauw, V. Everts, and W. Beertsen. Expression of integrins by human periodontal ligament and gingival fibroblasts and their involvement in fibroblast adhesion to enamel matrix-derived proteins. *Journal of periodontal research*, 37(5):317–323, 2002.

[299] S. M. Wahl, J. Swisher, N. McCartney-Francis, and W. Chen. Tgf-beta: the perpetrator of immune suppression by regulatory t cells and suicidal t cells. *Journal of leukocyte biology*, 76(1):15–24, 2004.

[300] B. Wang, B. M. Dolinski, N. Kikuchi, D. R. Leone, M. G. Peters, P. H. Weinreb, S. M. Violette, and D. M. Bissell. Role of alphav-beta6 integrin in acute biliary fibrosis. *Hepatology (Baltimore, Md.)*, 46(5):1404–1412, 2007.

[301] W. Wang, J. Xu, B. Du, and T. Kirsch. Role of the progressive ankylosis gene (ank) in cartilage mineralization. *Molecular and cellular biology*, 25(1):312–323, 2005.

[302] X. Wang, J. Hao, Y. Xie, Y. Sun, B. Hernandez, A. K. Yamoah, M. Prasad, Q. Zhu, J. Q. Feng, and C. Qin. Expression of fam20c in the osteogenesis and odontogenesis of mouse. *The journal of histochemistry and cytochemistry*, 58(11):957–967, 2010.

[303] X. Wang, J. Hao, Y. Xie, Y. Sun, B. Hernandez, A. K. Yamoah, M. Prasad, Q. Zhu, J. Q. Feng, and C. Qin. Expression of fam20c in the osteogenesis and odontogenesis of mouse. *The journal of histochemistry and cytochemistry*, 58(11):957–967, 2010.

[304] X. P. Wang, M. Suomalainen, S. Felszeghy, L. C. Zelarayan, M. T. Alonso, M. V. Plikus, R. L. Maas, C. M. Chuong, T. Schimmang, and I. Thesleff. An integrated gene regulatory network controls stem cell proliferation in teeth. *PLoS biology*, 5(6):e159, 2007.

[305] X. P. Wang, M. Suomalainen, C. J. Jorgez, M. M. Matzuk, S. Werner, and I. Thesleff. Follistatin regulates enamel patterning in mouse incisors by asymmetrically inhibiting bmp signaling and ameloblast differentiation. *Developmental cell*, 7(5):719–730, 2004.

[306] P. H. Weinreb, K. J. Simon, P. Rayhorn, W. J. Yang, D. R. Leone, B. M. Dolinski, B. R. Pearse, Y. Yokota, H. Kawakatsu, A. Atakilit, D. Sheppard, and S. M. Violette. Function-blocking integrin alphavbeta6 monoclonal antibodies: distinct ligand-mimetic and nonligand-mimetic classes. *The Journal of biological chemistry*, 279(17):17875–17887, 2004.

[307] C. H. Williams, T. Kajander, T. Hyypia, T. Jackson, D. Sheppard, and G. Stanway. Integrin alpha v beta 6 is an rgd-dependent receptor for coxsackievirus a9. *Journal of virology*, 78(13):6967–6973, 2004.

[308] P. J. Wipff and B. Hinz. Integrins and the activation of latent transforming growth factor beta1 - an intimate relationship. *European journal of cell biology*, 87(8-9):601–615, 2008.

[309] P. J. Wipff, D. B. Rifkin, J. J. Meister, and B. Hinz. Myofibroblast contraction activates latent tgf-beta1 from the extracellular matrix. *The Journal of cell biology*, 179(6):1311–1323, 2007.

[310] C. J. Witkop. Hereditary defects in enamel and dentin. *Acta Genetica et Statistica Medica*, 7(1):236–239, 1957.

[311] C. J. Witkop. Amelogenesis imperfecta, dentinogenesis imperfecta and dentin dysplasia revisited: problems in classification. *Journal of oral pathology*, 17(9-10):547–553, 1988.

[312] C. J. Witkop and J. J. Sauk. Heritable defects of enamel. *Oral Facial Genetics*, pages 151–226, 1976.

[313] J. J. Worthington, J. E. Klementowicz, and M. A. Travis. Tgfbeta: a sleeping giant awoken by integrins. *Trends in biochemical sciences*, 36(1):47–54, 2011.

[314] J. T. Wright. Oral manifestations in the epidermolysis bullosa spectrum. *Dermatologic clinics*, 28(1):159–164, 2010.

[315] J. T. Wright, B. Daly, D. Simmons, S. Hong, S. P. Hart, T. C. Hart, P. Atsawasuwan, and M. Yamauchi. Human enamel phenotype associated with amelogenesis imperfecta and a kallikrein-4 (g.2142g¿a) proteinase mutation. *European journal of oral sciences*, 114 Suppl 1:13–7; discussion 39–41, 379, 2006.

[316] J. T. Wright, P. S. Hart, M. J. Aldred, K. Seow, P. J. Crawford, S. P. Hong, C. W. Gibson, and T. C. Hart. Relationship of phenotype and genotype in x-linked amelogenesis imperfecta. *Connective tissue research*, 44 Suppl 1:72–78, 2003.

[317] J. T. Wright, T. C. Hart, P. S. Hart, D. Simmons, C. Suggs, B. Daley, J. Simmer, J. Hu, J. D. Bartlett, Y. Li, Z. A. Yuan, W. K. Seow, and C. W. Gibson. Human and mouse enamel phenotypes resulting from mutation or altered expression of amel, enam, mmp20 and klk4. *Cells, tissues, organs*, 189(1-4):224–229, 2009.

[318] J. T. Wright, M. Torain, K. Long, K. Seow, P. Crawford, M. J. Aldred, P. S. Hart, and T. C. Hart. Amelogenesis imperfecta: genotype-phenotype studies in 71 families. *Cells, tissues, organs*, 194(2-4):279–283, 2011.

[319] J. E. Wu and S. A. Santoro. Complex patterns of expression suggest extensive roles for the alpha 2 beta 1 integrin in murine development. *Developmental dynamics*, 199(4):292–314, 1994.

[320] Y. Xie, K. Gao, L. Hakkinen, and H. S. Larjava. Mice lacking beta6 integrin in skin show accelerated wound repair in dexamethasone impaired wound healing model. *Wound repair and regeneration*, 17(3):326–339, 2009.

[321] L. Xu, H. Harada, T. Yokohama-Tamaki, S. Matsumoto, J. Tanaka, and A. Taniguchi. Reuptake of extracellular amelogenin by dental epithelial cells results in increased levels of amelogenin mrna through enhanced mrna stabilization. *The Journal of biological chemistry*, 281(4):2257–2262, 27 2006.

[322] S. Yamada, K. M. Yamada, and K. E. Brown. Integrin regulatory switching in development: oscillation of beta 5 integrin mrna expression during epithelial-mesenchymal interactions in tooth development. *The International journal of developmental biology*, 38(3):553–556, 1994.

[323] P. M. Yamaguti, A. C. Acevedo, and L. M. de Paula. Rehabilitation of an adolescent with autosomal dominant amelogenesis imperfecta: case report. *Operative dentistry*, 31(2):266–272, 2006.

[324] Y. Yamakoshi, J. C. Hu, M. Fukae, F. Yamakoshi, and J. P. Simmer. How do enamelysin and kallikrein 4 process the 32-kda enamelin? *European journal of oral sciences*, 114 Suppl 1:45–51; discussion 93–5, 379–80, 2006.

[325] Y. Yamakoshi, T. Tanabe, S. Oida, C. C. Hu, J. P. Simmer, and M. Fukae. Calcium binding of enamel proteins and their derivatives with emphasis on the calcium-binding domain of porcine sheathlin. *Archives of Oral Biology*, 46(11):1005–1014, 2001.

[326] Z. Yang, Z. Mu, B. Dabovic, V. Jurukovski, D. Yu, J. Sung, X. Xiong, and J. S. Munger. Absence of integrin-mediated tgfbeta1 activation in vivo recapitulates the phenotype of tgfbeta1-null mice. *The Journal of cell biology*, 176(6):787–793, 2007.

[327] M. Yokozeki, E. Afanador, M. Nishi, K. Kaneko, H. Shimokawa, K. Yokote, C. Deng, K. Tsuchida, H. Sugino, and K. Moriyama. Smad3 is required for enamel biomineralization. *Biochemical and biophysical research communications*, 305(3):684–690, 2003.

[328] K. Yoshiba, N. Yoshiba, D. Aberdam, G. Meneguzzi, F. Perrin-Schmitt, C. Stoetzel, J. V. Ruch, and H. Lesot. Expression and

localization of laminin-5 subunits during mouse tooth development. *Developmental dynamics*, 211(2):164–176, 1998.

[329] T. Yoshida, J. Miyoshi, Y. Takai, and I. Thesleff. Cooperation of nectin-1 and nectin-3 is required for normal ameloblast function and crown shape development in mouse teeth. *Developmental dynamics*, 239(10):2558–2569, 2010.

[330] R. A. Young. Implications in atomic substitutions and other structural details in apatites. *J.Dent.Res.*, 53:193–203, 1974.

[331] Q. Yu and I. Stamenkovic. Cell surface-localized matrix metalloproteinase-9 proteolytically activates tgf-beta and promotes tumor invasion and angiogenesis. *Genes & development*, 14(2):163–176, 2000.

[332] R. Zaka and C. J. Williams. Role of the progressive ankylosis gene in cartilage mineralization. *Current opinion in rheumatology*, 18(2):181–186, 2006.

[333] Y. E. Zhang. Non-smad pathways in tgf-beta signaling. *Cell research*, 19(1):128–139, 2009.