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The Small Alternatively Spliced Amelogenin LRAP Modulates Early Stage Ameloblast Differentiation

by

Jonathan Stahl

DISSERTATION

Submitted in partial satisfaction of the requirements for

the degree of

DOCTOR OF PHILOSOPHY

in

Oral and Craniofacial Sciences

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GRADUATE DIVISION

of the

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by
Jonathan Stahl

Dedication and Acknowledgments

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Abstract

Amelogenesis is a regulated and sequential developmental cascade that results in expression of tissue specific gene products that form the enamel extracellular matrix. There remains considerable lack of knowledge regarding the precise mechanisms that control ameloblast differentiation and enamel matrix biomineralization. Amelogenins are the major protein product of ameloblasts and are comprised of multiple alternatively spliced isoforms that may function as structural molecules to regulate enamel crystal growth in addition to being signaling molecules that regulate cell differentiation. **Hypothesis:** The small alternatively spliced amelogenin known as leucine rich amelogenin peptide (LRAP) functions to modulate ameloblast differentiation from pre-ameloblasts to terminal differentiation. **Methods:** Transgenic mouse models that overexpressed LRAP in both a WT (TgLRAP) and an amelogenin null background (TgLRAP/AmelX Null) were examined to determine if this alternatively spliced protein had a direct effect *in vivo* on ameloblast differentiation by assaying histomorphology, gene expression, and protein expression patterns in comparison to wild-type and amelogenin null mice. Biomineralization was further assessed with microCT and von Kossa staining. *In vitro* primary ameloblast lineage cells were transfected with LRAP to study early developmental effects. **Results:** *In vivo* TgLRAP mice in the WT background showed a significant upregulation of enamel matrix gene products in pre-ameloblasts with earlier and greater amelogenin protein expression in pre-secretory and secretory ameloblasts. Apoptosis was increased in secretory and

transitional TgLRAP ameloblasts. Earlier mineral formation was also associated with the increased amelogenin expression. Downregulation of the master gene regulator SATB1 was also detected in pre-secretory and secretory ameloblasts. In the AmelX Null background TgLRAP overexpression did not lead to an observable phenotype compared to AmelX Null mice. *In vitro* primary cell culture demonstrated down regulation of developmental genes Notch1 and NfKappaβ1 was found. **Conclusions:** LRAP overexpression specifically modulates the expression of enamel matrix associated genes and proteins suggesting an earlier differentiation of ameloblasts from pre-ameloblasts to pre-secretory ameloblasts and an important role in regulating the timing of enamel matrix biomineralization.

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Chapter 1. Introduction

Amelogenins are the most abundant enamel matrix proteins comprising approximately 90% of all proteins secreted by ameloblast cells. As these proteins are cleaved and degraded, mineral deposition in the form of hydroxyapatite crystals occurs in a well-ordered pattern (Wen et al., 2001). It is thus believed that amelogenins play a major role in enamel biomineralization as they function to regulate the orientation, shape and length of enamel hyroxyapatite crystals (Fincham and Moradian-Oldak, 1993). The primary RNA transcript of amelogenin can be alternatively spliced to form at least 17 mRNAs, which when translated into proteins vary in abundance, with proportions that change during tooth formation (Hu et al., 1997b; Li et al., 1998; Simmer et al., 1994; Sire et al., 2005). Evidence suggests, however, that amelogenins do not merely function as tissue specific enamel related mineralization proteins. These proteins are believed to be multi-functional and among their more important functions is their ability to act as signaling molecules.

The objective of my thesis work was to determine what role LRAP plays in ameloblast differentiation. LRAP, or leucine rich amelogenin peptide, is a relatively plentiful spliced product that may possess growth factor like activity (Warotayanont et al., 2009; Wen et al., 2011). The exact function of LRAP in tooth formation remains unknown. The primary focus of my studies would involve characterizing the LRAP transgenic murine model in early development

to find clues as to what role LRAP plays in ameloblast differentiation and tooth formation. Gibson and co-workers originally developed the transgenic LRAP overexpressor mouse model (TgLRAP) (Chen et al., 2003), in which bovine LRAP expression is driven by the bovine amelogenin promotor, as part of a larger body of work to examine the potential of different amelogenin splice variants to rescue the amelogenin null phenotype. The developmental effects of LRAP overexpression at early stages of tooth formation were not characterized as part of the Gibson's lab previous work.

The significance of this investigation lies in the apparent potential that amelogenins hold in tissue repair, regeneration, and biomineralization. The overall goal of this proposal is to identify the effects that the small alternatively spliced amelogenin, which is hydrolyzed by MMP-20 to form Leucine Rich Amelogenin Peptide (LRAP) has in ameloblast differentiation. Both the originally secreted 59 amino acid alternatively spliced protein and the shortened, hydrolyzed 46 amino acid fragment isolated from the enamel matrix have been referred to as LRAP. In this study, we will refer to LRAP as the original uncleaved alternatively spliced protein. Evidence suggests LRAP does not function as a structural protein to promote enamel formation (Habelitz et al., 2006). I hypothesize that LRAP acts to control ameloblast differentiation.

In these studies, I set out to determine the regulatory role of the small polypeptide in enamel organ development. These studies began with my

characterizing the phenotype of the LRAP overexpression mouse model (TgLRAP), in comparison to wild type and other mouse models. I firstly characterized and compared mouse molars with when LRAP is overexpresed in a wild type and an amelogenin null background. Secondly, I analyzed expression markers unique to different stages of ameloblast differentiation, to determine if overexpression of LRAP alters biological mechanisms related to ameloblast differentiation. In additional studies I developed cell culture models to further investigate LRAP function and used LRAP overexpression compare the molar and incisor tooth organ models.

Chapter 2. Background

I. Amelogenesis

Amelogenesis is a complex and regulated process initiated through interactions of the ectodermal derived epithelial and neural crest derived mesenchymal cell layers in the tooth organ. It is signaling between the pre-ameloblasts and pre-odontoblasts, that initiates the final series of reciprocal signaling resulting in cytodifferentiation (Thesleff, 2006). Differentiation of dental epithelial cells to ameloblasts follows secretion of the underlying dentin matrix by odontoblasts. Soon after the deposition of the first layer of dentin, odontoblasts express matrix metalloproteases (MMPs) to digest the basement membrane and allow enamel and dentin matrices as well as their respective mineral phases to come into direct contact, ultimately forming a strong mechanical bond between enamel and dentin (Smith, 1998).

As cells of the inner enamel epithelium begin to differentiate into ameloblasts, they elongate and their nuclei shift distally away from the dental papilla (precursor of the dental pulp) (Zeichner-David et al., 1995). Immediately after dentin matrix is deposited cuboidal pre-ameloblasts elongate and form a monolayer of pre-secretory stage ameloblasts, which polarize and express enamel matrix proteins and then lay down the enamel matrix in the secretory stage (Fig 1). Mineralization and initial matrix protein hydrolysis begins at this stage. The matrix proteins are secreted at a mineralization front through a specialized apical extension called a Tomes' process, which is the only portion of

the ameloblast in direct contact with the enamel matrix. After deposition of the matrix is completed the columnar ameloblasts undergo functional and morphological changes back to a shortened columnar transitional stage and then to cuboidal cells considered maturation stage ameloblasts. During the maturation stage the remaining organic matrix material while mineralization of the enamel moves toward completion (Hu et al., 2007; Smith and Nanci, 1995).

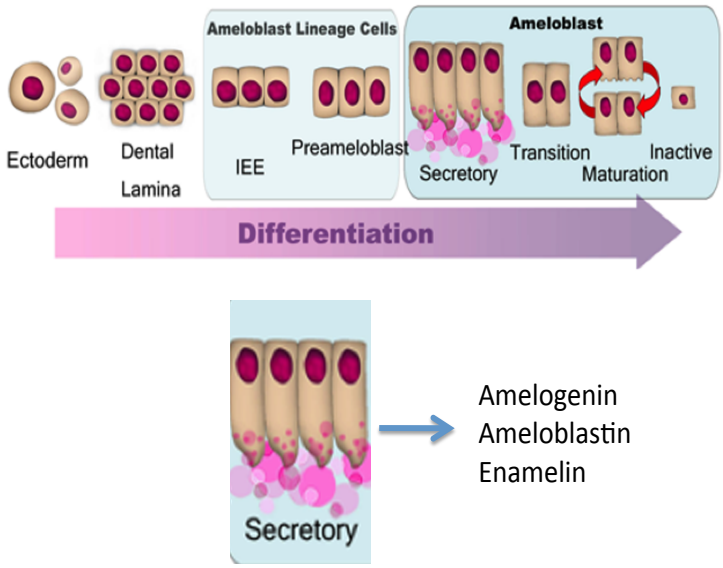

Figure 1. Stages of Ameloblast Differentiation. Ameloblasts are derived from the oral ectoderm cell, which proceed through several distinct stages of differentiation each characterized by a different morphology. Cuboidal pre-ameloblasts elongate slightly to become pre-secretory ameloblasts capable of producing enamel matrix proteins, but not secreting them. Further elongation leads to secretory stage ameloblasts which bexome polarized orienting their apical end towards the newly formed dentin. Extracellular deposition of amelogenin occurs through a specialized distal apical extension known as a Tomes' process. After the matrix is laid down ameloblasts transition and shorten to a maturation stage during which mineralization of the enamel is completed and hydrolysis of the matrix proteins occurs.

The removal of amelogenins from the enamel matrix directs matrix mineralization and creates space for the hydroxyapatite crystals to expand in width and thickness (Lu et al., 2008; Yamakoshi et al., 2011). Amelogenin hydrolysis is well controlled and requires specific proteases whose spatiotemporal expression must also be exquisitely regulated. Matrix metalloproteinase-20 (MMP20) is secreted during the secretory stage and initiates amelogenin hydrolyis. During maturation, when ameloblasts modulate between smooth ended and ruffle-ended ameloblasts, kallikrein-related peptidase 4 (KLK4) is secreted to complete amelogenin hydrolysis while the ameloblasts remove the hydrolyzed amelogenin fragments. The ameloblasts then lose polarity and form and epithelial sheet referred to as the reduced enamel organ, which is lost upon tooth eruption (Schroeder and Listgarten, 1971).

Although the enamel matrix is chiefly composed of amelogenins other proteins involved in enamel formation include ameloblastin, enamelin, and amelotin. The function of these proteins is not well-understood. Ameloblastin is thought to function as a cell-attachment protein (Zhang et al., 2011a; Zhang et al., 2011b). Enamelin is believed to function as a necessary component of the mineralization front that establishes mineral ribbons during the secretory stage (Fan et al., 2008). Mice which fail to express ameloblastin or enamelin do not form a normal enamel layer. Mice in which amelotin has been overexpressed, fail to develop Tomes' process and secrete a functional matrix (Lacruz et al., 2012).

The inorganic or mineral content of tooth enamel is a highly substituted form of hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, commonly referred to as carbonated hydroxyapatite (Young, 1974). The first formed enamel is a rodless enamel as the proximal end of the newly formed ameloblast facing the odontoblast is still flat. After this first rodless enamel layer is formed, the ameloblasts migrate away from dentin surface which permits the formation of the aforementioned conical projections called Tomes' processes, and gives the adjacent ameloblast cells a saw-toothed appearance. Ameloblasts are interconnected by junctional complexes, and alternating rows of ameloblasts are hypothesized move past each other as enamel rods are formed (Skobe and Stern, 1978). This, in effect, leads to ameloblasts forming a unique mineralized structure with a decussating pattern made up of rows of interwoven enamel rods. With the formation of Tomes' processes, the enamel matrix transitions from aprismatic (rodless) enamel, to form enamel matrix in the form of rods or prisms that become highly mineralized (Lacruz et al., 2013; Simmer and Fincham, 1995). This is followed by enamel maturation. Mature dental enamel is acellular, is composed of more than 95% mineral in the form of carbonated hyrdroxyapatite, contains less than 1% organic material, contains no collagen (Fincham et al., 1999; Robinson et al., 1998), and is incapable of biological repair.

Ameloblasts function to resorb much of the water and organic matrix from enamel in order to provide space for the growing enamel crystals. How calcium moves from the blood vessels through the enamel organ to reach enamel is not a

well understood process, but likely involves intercellular and trancellular routes. Recent biochemical findings have led to suggestions that calcium is transported through ameloblasts in high capacity stores associated with the endoplasmic reticulum, avoiding the cellular effects that would result from excess calcium in the cytoplasm (Lacruz et al., 2013).

II *Alternative splicing of amelogenins*

Alternative splicing is a key event in eukaryotes that increases the coding capacity of the human genome allowing for one gene to produce multiple proteins with potentially different functions. For the vast majority of eukaryotic genes the protein coding regions exist as short segments known as exons, which are separated by intervening sequences called introns (Sharp, 1987). Following transcription of both exons and introns into primary RNA from genomic DNA the intervening sequences are removed by the process of RNA splicing in which the exons are joined to produce mRNA, which can be translated into protein. In the process known as alternative splicing, the primary intron-containing RNA transcript of a gene is spliced in two or more different ways resulting in different combinations of exons within the resulting mRNA. Alternative splicing represents a highly flexible means of generating protein diversity from a single gene producing distinct but related protein products with potentially different functions (Breitbart et al., 1987). It increases the coding capacity of the eukaryotic

genome, effectively increasing diversity of functional proteins and signaling network complexity. Alternative splicing is especially prevalent in terminally differentiated non-dividing cells such as ameloblasts, as gene regulation at the transcriptional level would be difficult (Hammes et al., 1994; Pironcheva and Russev, 1997).

Amelogenins are highly conserved amongst divergent species. Both amino acid and cDNA sequences for bovine, human, mouse and porcine amelogenins have demonstrated greater than 80% conservation (Deutsch, 1989). Amelogenin has been known for over 30 years to constitute the major protein portion of the forming dental enamel matrix, and many studies have been undertaken to understand its biochemical function during enamel formation and mineralization. It is well established that secreted amelogenins form a heterogeneous population of molecules that serve as a template for deposition of mineral in the enamel matrix. Much of this heterogeneity can be attributed to extracellular processing of one or more parent molecules. Additionally, heterogeneity results from alternative splicing of amelogenin mRNAs. The majority of the amelogenin protein isoforms are in the 5 to 30 kDa range.

Amelogenins comprise greater than 90% of the organic proteins in the developing organic matrix. The murine gene produces at least 17 messages through alternative splicing. Since the initial amino acid sequence of the processed secreted amelogenins is so highly conserved, numbering the residues

generally begins with N-terminal methionine as residue number 1 (Fig 2a). Exon 1 contains an untranslated region of the mature mRNA. Exon 2 contains the remainder of the 5'- untranslated region, followed by the initiation codon, then the sequence coding for the signal peptide, and finally the first two amino acids of the secreted protein.

Amelogenin proteins formed from various splice variants are identified by the total number of amino acid residues in the secreted product. Thus amelogenin isoforms are frequently referred to by the predicted number of amino-acids contained in the secreted protein; for example M180 contains 180 amino-acids, M194 includes exon 4 and contains 194 amino-acids, and LRAP contains 59 amino-acids and is often referred to as M59 (Veis, 2003), and so on. The various protein isoforms are predicted to perform different functions in the development of dental enamel, such as structural and regulatory roles, as well as intercellular signaling (Termine et al., 1980). Each splice site is located so that the reading frame is maintained, and the N and C termini of amelogenin proteins are constant with the C termini ending either at exon 7 (Fig 2b-c), or an alternative C terminus in rodents formed as a result of exon 7 being skipped and exons 8&9 (Fig 2e) constituting a C terminus with the addition of 24 exons (Li et al., 1998).

Why rodents express exons 8&9 remains unknown, but its possible that these two extra exons may relate to controlling the growth of the continuously erupting incisor in rodents as they are not present in other species. Exon 2 contains the

start codon and is always present, however, rodent exon 3, which contains the sole phosphorylated residue, Ser 16, can be skipped.

There is also no data describing the precise relative prevalence of the majority of these amelogenin alternatively-spliced isoforms, as most have been identified by sequencing RT-PCR DNA products. However, the most abundant amelogenin variants consists of exons 1, 2, 3, 5, 6 and 7 (Fig 2b-c) and leucine rich amelogenin peptide (LRAP), which lacks the exon 6abc domains in addition to exon 4 (Fig 2d & 3). The amelogenin splice junctions for exons 1, 2, 3, 4, 5 and 7 are predictable based on DNA sequence, the so-called intronic dinucleotides GT and AG at the 5' and 3' splice sites respectively (Veis, 2003), however exon 6 contains three cryptic splice junctions, and these sites divide exon 6 into four regions that have been defined as exons 6A, 6B, 6C and 6D (Simmer and Snead, 1995; Veis, 2003). Exon 4 is frequently skipped (Fig 2d). Regulatory mechanisms of amelogenin splice site choice remains poorly understood.

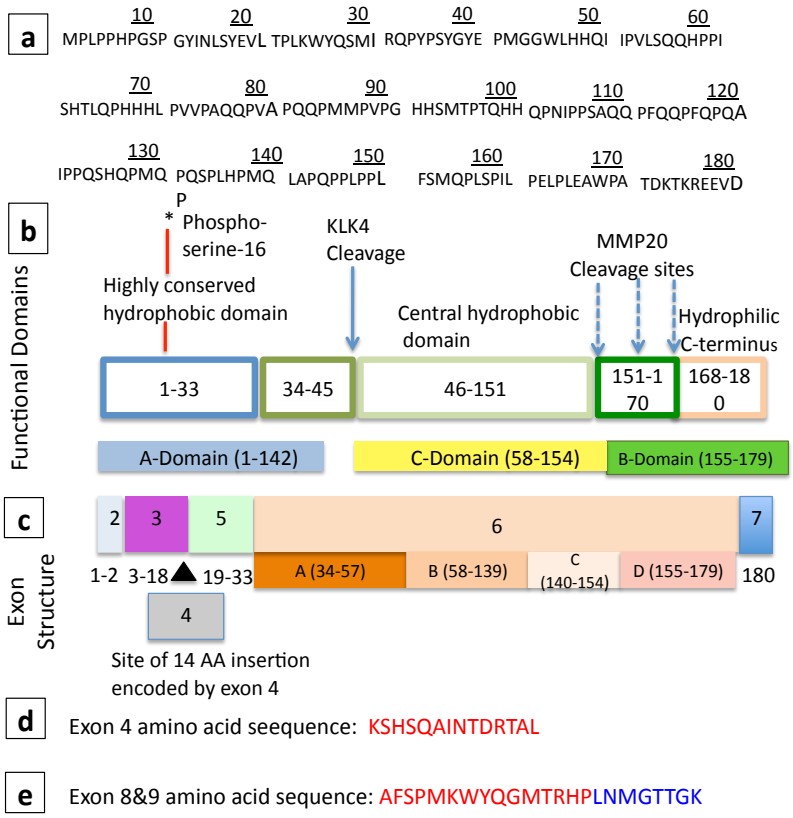

Figure 2: Domains and exon structure of secreted full-length (M180) murine amelogenin. (a) Amino acid sequence (based on amelogenin isoform 1, accession number P63277). (b) Functional domains. The red line above A-domain indicates the single phosphorylation site (serine 16), which is located in exon 3. The major cleavage sites for MMP20 and KLK4 are indicated with blue dashed arrows. The N-terminal A-domain is cleaved off by KLK4. The B-domain includes the 13 amino acid C-terminus which is hydrophilic and suggested to bind hydroxyapatite. (c) Exon structure. Includes exons 2-7. Exon 1 is not included because it lies 5' of the start codon for translation. (d) Sequence of the 14 amino acid exon 4. (e) Exon 7 can be skipped with exons 8-9 alternatively constituting the c-terminus.

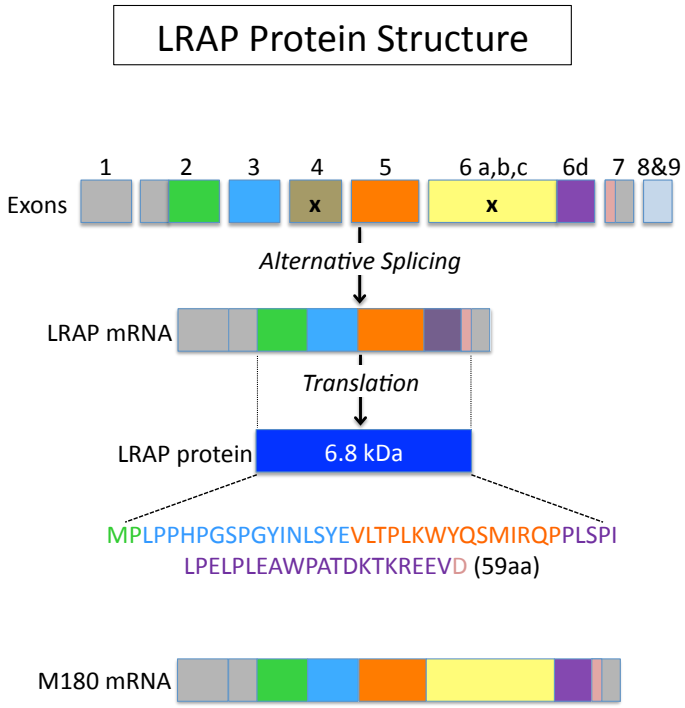

Figure 3. Alternative spicing pattern of human LRAP and its amino acid sequence. Murine LRAP is encoded by exons 2, 3, 5, 6d (3' end of exon 6) and 7, resulting in a 59 amino acid residue, whose sequence is identical to the first 33 and last 26 residues of the full amelogenin (M180). Thus, LRAP is identical at the N and C-terminus domains, but lacks the large central hydrophobic domain of exon 6 (indicated by X). An alternative stop codon can occur where exon 7 is skipped and two additional stop codons (exons 8&9) are added.

For rodents (mice and rats) amelogenin is transcribed from a single gene on the X chromosome. Initially, the mRNA isolated from mice was reported to contain seven exons, with the predominant spliced isoform lacking exon 4. Other splice patterns include deletion of exons 3,4, and 5 all of which are small in size and variations in the size of exon 6 by alternative splicing. Following these initial reports, an additional 2 exons 8 and 9 were identified in rodents (Li et al., 1998).

For humans amelogenins codes on both the X (AMELX) and Y (AMELY) chromosomes in males with Y estimated to be only about 10% as active (Salido et al., 1992). Human, murine, and bovine X-chromosome linked amelogenin gene structure is identical in coding sequence from the N-terminus until the middle of exon 5. Exon 4 is reported in an in silico analysis of bovine, murine and human sequences (Sire, 2012)

Alternative splicing of amelogenin mRNA is partially explained by examination of mouse amelogenin cDNA. There exist a total of 21 potential 3' internal acceptor sites within exon 6 alone, owing to the high incidence of the glutamine codon CAG, which provides potential splice consensus signals (Gibson, 1999). At present it is not possible to say whether each individual alternatively spliced molecule is of specific functional importance in odontogenesis. Studies have reported great variations in production of various isoforms but they may be temporally and spatially regulated, with less abundantly expressed isoforms having specific roles outside that of the more abundant major amelogenin

transcript. Conversely, alternatively spliced amelogenins may be due to *in vivo* splicing artifacts that produce 'ghost' genes with no function that do not adversely affect matrix function.

Regulation of splice site choice leads to differences being generated during development in the amelogenin protein, which provides a mechanism for generating variations in enamel structure. Exon 4 is frequently included in mouse and sometimes human, but not in bovine mRNAs (Hu et al., 1997b; Yuan et al., 1996). Exon 6 has several alternative 5' splice sites in mice, but no more than one alternative 5' site has been found in other species (Salido et al., 1992; Simmer et al., 1994). Splicing of exon 6 can delete the internal hydrophobic domain of exon which is involved in the formation of protein aggregates thought to be critical in enamel crystal formation (Gibson, 1999; Wen et al., 1999). This may explain why LRAP, which lacks the hydrophobic segment of exon 6, does not function as a structural protein in biomineralization.

Exon4 consists of a hydrophilic sequence of 14 charged polar amino acids devoid of the high proline content that characterizes amelogenin. The polar character of the exon suggests that amelogenin isoforms that contain the sequence for exon4 may fold to expose this sequence on the surface of the protein. This may alter the binding specificity for other enamel matrix or mineral components (Hu et al., 1997b) Several studies have shown that recombinant LRAP protein with the exon 4 sequence (m73) has functions that differ from

LRAP without exon4. These studies all focused on exon4's effects on odontogenic and non-odontogenic mesenchymal cells.

Amelogenins exhibit an unusual amino acid composition. Proline accounts for around 25-30% of the amino acids present and there are also relatively high levels of histidine, glutamine and leucine (Eastoe, 1964), allowing for considerable hydrophobicity of the molecule. The prolines are thought to inhibit the formation of secondary structures such as ß-sheet, α-helix, and random coil, producing an intrinsically disordered protein. This disorder is believed to allow amelogenins molecules to self-assemble into the hydrophobic supramolecular monodisperse structures known as nanospheres (Fincham et al., 1994). In the mineralizing enamel matrix nanospheres are thought to bind to hydroxyapatite crystals and control crystal growth (Lyngstadaas et al., 1995)
(Moradian-Oldak, 2001).

The newly secreted amelogenin are thought to aggregate into ~20nm hydrophobic nanospheres and the nanospheres interact electrostatically parallel with the c-axis of the forming hydroxyapatite crystals to keep individual forming rods from fusing together and are hypothesized to act as spacers to direct crystal growth. In an *in vitro* study by Habelitz et al using recombinant protein seeded onto glass ceramics both LRAP and rH175 (full-length human amelogenin) assembled into nanospheres. Apatite crystal growth, however, was not significantly affected by LRAP, while rH175 accelerated crystal growth by up to

50-fold. Full-length amelogenin thus may play a key role in the structural organization of the mineral within fully formed enamel and may also regulate the nucleation and growth pattern of the enamel hydroxyapatite crystals LRAP has other functional roles.

A larger question to consider is why there is such a high level of alternative splicing of amelogenin. Since amelogenin is reported to have both structural and signaling roles, alternative splicing may separate these functions temporally and spatially during development.

III *Amelogenin as signaling molecules*

A major emphasis of studies of amelogenin proteins have been focused on understanding its functions in controlling mineralization of enamel. Increasingly, however, new lines of investigation have been developing that lead to the suggestion that various amelogenin splice products might function as signaling molecules with roles not only in amelogenesis, but also in development of other hard and soft tissue types.

Clinically the cell signaling functions of amelogenins has been proposed as an explanation for the therapeutic effects of the commercial product Emdogain® which is composed of enamel matrix derivative from extracted from developing porcine enamel and is rich in amelogenins (Hammarstrom, 1997; Hammarstrom

et al., 1997). Use of Emdogain in periodontal defects has resulted in significant gains in both the periodontal attachment complex and alveolar bone growth (Jepsen et al., 2004; Sculean et al., 2004). Amelogenins have even been shown in both *in vitro* and *in vivo* studies to be effective in healing wounds outside the oral cavity such as hard-to-heal venous leg ulcers (Romanelli et al., 2008).

Emdogain was recently separated into low-molecular-weight (<6kDa) and high-molecular weight(>6kDa) fractions and the higher fractions which included LRAP was found to promote osteogenic differentiation in PDL and bone progenitor cells whereas the lower fraction which included TRAP (tyrosine rich amelogenin peptide) suppressed bone formation (Amin et al., 2012). The authors of this study further created a synthetic peptides to TRAP and LRAP and found similar to the EMD fractions that LRAP promoted osteogenic differentiation and mineral nodule formation, but TRAP did not. This study did not look at the effects of full-length amelogenin.

Over forty years ago Urist and co-workers was demonstrated that bone could form when cell-free demineralized dentin was implanted intramuscularly into rats (Bang and Urist, 1967). Rat incisor dentin was isolated and separated into protein fraction with chromatography. A fraction in the 6-10 kDa range was capable of inducing rat muscle fibroblasts to undergo chondrogenic transformation *in vitro* as evidenced by production of collagen II (Boskey et al., 1990; Koskinen et al., 1985). The sequence of the fragment excluded the

possibility that either BMP or TGF-beta was responsible for the result. Further analysis showed that the chondrogenic inducing activity was the result of a low molecular weight amelogenin. The origin of the amelogenin peptide was subsequently identified through PCR using a rat odontoblast cDNA library to have two possible sequences comprising exons 2, 3, 5, 6d, and 7 and exons 2, 3, 4, 6d, and 7 with molecular masses of 6.7 kDa and 8.1 kDa respectively (Nebgen et al., 1999). Interestingly, these authors determined that the two mRNAs from the odontoblast cDNA library expressed almost equally, differing from developing rat enamel organ in which exon 4 is less abundantly expressed (Salido et al., 1992).

Veis et al. (Veis et al., 2000), set out to determine whether low molecular mass amelogenin-related polypeptides based on sequences identified in a odontoblast cDNA library, can affect differentiation pathway of embryonic muscle fibroblasts in culture and lead to the formation of mineralized matrix in *in vivo* implants. His group produced two exogenous peptides designated r[A+4] (8.1 kDa) and r[A−4] (6.9 kDa) based respectively on amelogenin exons 2,3,4,5,6d,7 and 2,3,5,6d,7.

In the EMF culture system, the [A−4] peptide up-regulated transcription factor Cbfa1 related to osteogenesis, whereas [A+4] more prominently up-regulated factor Sox9 related to chondrogenesis (Veis et al., 2000). *In vivo* ectopic implants into rat quadricep muscle were performed using a polyactide-polyglycolide carrier again containing either LRAP plus exon 4 (r[A+4]) or LRAP

minus exon 4 (r[A-4]). The results showed that both were active in inducing cellular growth into the implants, followed by extracellular matrix production, vascularization, and mineralization after about 4-weeks (Veis et al., 2000).

Differences were seen between the two peptides in that LRAP minus exon 4 produced a more pronounced and diffuse mineral deposition than LRAP plus exon 4 which tended to produce more focal and highly vascularized mineral deposits (Goldberg et al., 2003). Thus, these authors concluded that the cell signaling activities of the amelogenin peptides resulted in the differentiation formation of mineralized tissues.

Other research explored what effects [A-4] and [A+4] would have on the pulpal dentin complex of a maxillary molar in which the pulp was intentionally exposed and treated with specific amelogenin peptides. Goldberg *et al*. looked at the effects of capping the pulp with agarose beads soaked with either [A+4] or [A-4] and implanted in cavities drilled through to the pulp of rat molars. With [A+4], mineralization occurred in the crown part of the pulp and a dentinal bridge closed the cavity within 15 days. Coronal reparative dentin was seen in [A-4] implants, but the major effect was that the mesial root canal became filled with reparative dentin by day 30 post implantation. This would suggest that LRAP either with our without exon 4 transcripts, has the potential to be used in tissue engineering reparative dentin.

Mechanistically, it was shown that both splice products increase odontoblast proliferation, with [A-4] inducing an osteogenic phenotype in some cells more quickly (Lacerda-Pinheiro et al., 2006). A later study showed that *in vivo* recombinant LRAP containing exons 8&9 (LRAP 8, 9) further enhanced reparative dentin formation as compared to LRAP (A-4). *In vitro* LRAP 8, 9 promoted dental pulp stem cell proliferation and differentiation to a greater extent than LRAP (Huang et al., 2012). These data suggest that amelogenin exons 8 and 9 may be useful in amelogenin-mediated pulp repair.

Cementum, which covers the tooth root surface is a mineralized tissue critical to attaching the root to the surrounding alveolar bone; however, control of its formation remains poorly understood. One hypothesis is that enamel matrix proteins/peptides secreted by ameloblasts and/or epithelial rest cells contribute to the control of cementum formation. Based on this, Boabaid et al. tested whether the addition of exogenous LRAP to a cementoblast cell line (OCCM-30) would affect cementogenesis (Boabaid et al., 2004). They determined that the addition of LRAP resulted in a down regulation of osteocalcin and up regulation of osteopontin (OPN) in a dose- and time-response fashion, and inhibited the capacity of mineral nodule formation. Transcripts for osteoprotegerin were increased in LRAP-treated cells compared to control, but receptor activator of nuclear factor kappa-B ligand (RANKL) mRNA levels were not affected. Core binding factor alpha (Cbfa) mRNA, expressed constitutively, was not affected by LRAP. Signaling pathway assays suggested involvement of the MAPK pathway,

since the addition of the MAPK inhibitor suppressed OPN expression in LRAP-treated cells.

Studies by Snead and co-workers demonstrated that LRAP activates the canonical Wnt signaling pathway to induce osteogenic differentiation of mouse embryonic stem (ES) cells through the regulation of Wnt agonists and antagonists (Warotayanont et al., 2009; Warotayanont et al., 2008). Further work by the same group found that LRAP activates Wnt signaling, through the upregulation of Wnt10b, to promote osteogenesis of mesenchymal stem cells at the expense of adipogenesis when using the bipotential bone marrow stromal cell line ST2. LRAP has the potential to take advantage of the therapeutic value of Wnt signaling pathways without direct application of Wnt proteins, thereby eliminating the potential adverse effect on cells of direct Wnt protein exposure. Compared to the glycosylated Wnt proteins (over 40 kDa), LRAP is much smaller, giving LRAP a clear advantage as a small molecule for delivery as a therapeutic agent (Wen et al., 2011).

Another possible signaling role of amelogenins begins early in tooth development and relates to their possible role in epithelial-mesenchymal interactions between developing ameloblasts and odontoblasts. Tooth development is regulated at all stages by complex reciprocal paracrine and autocrine signaling communications between inner enamel epithelium (IEE) and the dental mesenchyme (Thesleff, 2006). Signals from the pre-odontoblasts lead the pre-ameloblasts to leave the

proliferative phase and commit toward differentiation. In turn, signals from pre-ameloblasts may commit pre-odontoblasts to differentiate into odontoblasts capable of laying down a pre-dentin matrix. Molecules in the pre-dentin matrix further instruct differentiation of the ameloblasts, which leads to extension of the specialized distal apical Tomes' process which begins laying down enamel matrix proteins on the pre-dentin matrix. Ameloblast cellular polarity is established through the deposition of the ameloblast extracellular matrix into the space that surrounds the Tomes' process and abuts the forming dentin layer (Smith, 1998).

Amelogenins, which have been shown to be transiently synthesized by odontoblasts, might have a role in the early signaling leading to development of pre-odontoblasts or in sequential and reciprocal signaling events that occurs between pre-odontoblasts and pre-ameloblasts

Further more, splice variants containing exon 4 may have a specific role in odontogenesis and signaling from odontoblasts to ameloblasts. Veis and colleagues (Iacob and Veis, 2006) dissected mouse lower first molars and cultured them in the presence of either LRAP with or without exon 4. Odontoblast differentiation was induced in the presence of LRAP with exon 4 as evidenced by an increased expression of dentin matrix protein 2 (DMP2) in both differentiating odontoblasts and pre-ameloblasts. LRAP lacking exon 4 showed an inhibitory effect on ameloblast differentiation.

Several studies explored the effects of adding exogenous LRAP to ameloblast lineage cells. Le et al. (Le et al., 2007) added recombinant human LRAP to human enamel organ epithelial cells. They found LRAP had no effect on cellular proliferation, but did effect cellular differentiation as observed by an up-regulation of amelogenin synthesis down-regulation in Notch1 expression. Iacob and Veis (Iacob and Veis, 2008) added exogenous recombinant LRAP to the murine enamel organ epithelial cell line LS8 and also found that cellular differentiation was affected. Specifically, they reported a 13.8 fold increase in M180 expression using quantitative PCR. Their data further suggested that LRAP modulated M180 RNA expression through the nitric oxide (NO) signaling pathway as the pathway marker NOS2 was significantly upregulated after treatment with LRAP and blockage of the pathway using an inhibitor (iNOS) concurrently with LRAP, lead to a decrease in M180 expression.

The signaling activity of any amelogenin isoform will depend on its interaction with a specific receptor. Protein sequence of the specific isoform will determine secondary and tertiary structure that affects receptor-ligand binding. The three dimensional structure of amelogenin has not been solved by crystallographic methods. As mentioned above, due to the high percentage of proline residues it has been suggested that amelogenin is an intrinsically disordered protein (IDP) (Delak et al., 2009). IDPs are proteins without a folded structure under physiological conditions, being enriched in proline residues (Tompa, 2002). IDPs can potentially have multiple binding partners or functions due to their structurally

plastic nature, and can be involved in signaling pathways (Wright and Dyson, 2009).

LAMP1

Evidence that amelogenin functions in a cell signaling role was suggested by the characterization of a plasma membrane binding protein that has been reported to be a cell-surface receptor. Two amelogenin splice products (M73 and M59, LRAP with and without Exon 4) have been shown in fibroblasts to bind with saturation kinetics to a 95 kDa transmembrane protein identified as lysosomal-associated membrane protein-1 [LAMP-1] (Tompkins et al., 2006). LAMP1 immunoreactivity is observed at the plasma membrane of most cell types (Hunziker and Geuze, 1996; Kannan et al., 1996). LAMP 1 may function as a specific endocytosing ligand binding receptor for LRAP, and may also be involved in the trafficking of LRAP to late endosomes or lysosomes (Xu et al., 2008). Previous studies have shown that LAMP 1 co-localizes to internalized small amelogenin protein, LRAP, in the perinuclear region of cells (Tompkins et al., 2006; Zou et al., 2007). A previous study reported that LAMP1 is expressed by ameloblasts at all stages of amelogenesis, and extracellular Emdogain® of which amelogenin is the major component is transferred into the cell cytoplasm by direct passage of amelogenin into LAMP1 positive vesicles (Shapiro et al., 2007).

It has not yet been determined what downstream signals are mediated by LAMP-1 and LRAP. The presence of LAMP1 in ameloblasts is suggestive of a functional role for LAMP1 in enamel formation, but this statement is made with the understanding that LAMP1 has a ubiquitous tissue expression profile (Andrejewski et al., 1999) and that the presence of LAMP1 in ameloblasts was expected.

Although LAMP1 has been shown to be a binding partner for LRAP it is not known to be part of any actual signaling pathway. Lamp1 may function by binding to and restraining amelogenin in a specific structure by which the protein could then bind correctly to a receptor, which initiates an intracellular signaling cascade. As to why LRAP plays a role specifically in signaling and not on mineralization it has been hypothesized that the hydrophobicity displayed as a result of the high proline content of the 6abc domain contributes to the strong aggregation properties of amelogenins that leads to nanosphere formation, which is believed to be critical for enamel formation (Du et al., 2005).

Studies using the addition of exogenous recombinant proteins have several potential drawbacks with regards to amelogenins. One problem is that recombinant proteins have been spared post- translational modifications that might occur *in vivo,* though amelogenins have only one post-translational modification resulting in phosphorylation of a serine on exon 3. Another concern is that during their purification, the peptides are exposed to denaturing solvents

and may be in a non-native folded or non-native aggregated state.

IV. *Animal Models to study amelogenin function*

The amelogenin null mouse line was made by cloning a segment of the murine amelogenin gene lacking base pairs coding for the signal peptide and part of intron 2, into an expression vector. For clone selection, an antibiotic resistance gene expressed in the opposite direction, was cloned in front of the truncated amelogenin. The vector was transferred into ES cells and selected clones were injected into blastocysts to produce variable off spring with germ line transmission. The shortened RNA did not translate any amelogenin protein (Gibson et al., 2001).

The amelogenin knockout mouse tooth is covered with a thin layer of enamel, about 10-20% of normal enamel thickness, with a disorganized enamel structure. The enamel lacks discernable enamel rod formation and normal decussation patterns. Though amelogenins are not secreted, other enamel matrix proteins, including enamelin and ameloblastin are present and presumably contribute to the formation of the thinly mineralized matrix (Gibson et al., 2001). This phenotype is similar to some tooth phenotypes found in humans from families with X-linked amelogenesis imperfecta resulting from AMELX gene mutations (Wright et al., 2003).

An LRAP overexpression transgeninc mouse (TgLRAP) was constructed using

bovine LRAP cDNA. The vector included 5.5 kb of bovine X-chromosomal upstream sequence, untranslated exon 1, intron1, the remaining LRAP cDNA and 500 bp of 3' genomic sequence (Gibson et al., 1991a). Transgenic mice were produced by microinjection of the insert from the expression vector into fertilized murine eggs. The bovine sequence differs from the murine sequence by multiple base pairs, and therefore this construct allows the transgene to be detected by PCR using bovine-specific primers. PCR primers were designed to produce a 531 bp product from tail DNA, which were used to identify transgenic mice (Appendix 1).

Enamel thickness and structure from the TgLRAP mouse incisor was reported as relatively normal (Chen et al., 2003). Detection of the transgene was primarily limited to the molar. Nanoindentation revealed no differences between LRAP transgenic and wild-type murine enamel.

The TgLRAP mice were crossed with amelogenin null mice to determine if LRAP could rescue the amelogenin null phenotype. The AMELnull/TgLRAP mice had hypoplastic enamel that lacked normal prism structure and did not show signs of rescue of the previously describe amelogenin null phenotype. MicroCT, showed that enamel volume and density were similar to those from KO mice. However, in etched samples examined by SEM, an organization of the enamel rod pattern was observed in the LRAP transgene in that there was an organization in the enamel rod structure not present in the KO mice alone indicating a partial rescue

may have resulted (Gibson et al., 2009).

Using a similar approach as they did in developing the TgLRAP model Gibson and co-workers (Chen et al., 2003) also developed a transgenic mouse that overexpressed the full-length (exons 1,2,3,5,6,7) variant of amelogenin known as M180. An expression vector which included 5.5 kb of bovine AmelX upstream genomic sequence, bovine AmelX untranlsated exon 1 and intron 1, and exons from either bovine or murine AmelX cDNA. The bovine DNA sequence of the overexpressed full length amelogenin (M180) varied from the native murine amelogenin by 3 amino acids to allow for specific PCR primer analysis, but the bovine substitutions to the murine sequence were not in highly conserved areas. After injection strains with germ-line transmission were developed and their phenotype lacked any obvious enamel defects and was indistinguishable from WT enamel (Gibson et al., 2007).

The function of M180 in the mouse model was explored by mating the mouse with an AmelX null mouse. The male TgM180 was mated with a female AmelX null mouse as amelogenin is sex linked in mice to the X chromosome. TgM180KO mice demonstrated improvement in their enamel thickness and greater enamel density and volume based on microCT compared to the null mouse alone and was considered a partial rescue (Gibson et al., 2007). Hardness and elastic modulus were not the same as WT mice, and this was suggested to be due to defective organization of the enamel structure.

A further study was conducted which explored if additional rescue of the amelogenin null phenotype would occur if an amelogenin null mouse was double crossed with both a mouse overexpressing M180 and a mouse overexpressing LRAP (Gibson et al., 2011). The addition of LRAP to the M180 increased enamel thickness and lead to enhanced development of decussating patterns of enamel of molars. It is unclear whether the dual transgene overexpression improvement in enamel properties induced by LRAP was related to signaling or improvement in structural properties. Failure to have a complete rescue of enamel may relate to the importance of other amelogenin isoforms in enamel development.

The focus of these published studies was to determine the role of individual amelogenin isoforms on enamel matrix formation. In the studies completed for this thesis, I addressed the hypothesis that LRAP has a signaling role in directing ameloblast differentiation.

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Chapter 3. Published Manuscript

Leucine rich amelogenin peptide alters ameloblast differentiation *in vivo*

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Abbreviations: LRAP, leucine rich amelogenin peptide; MMP20, matrix metalloproteinase

Abstract

Highly mineralized tooth enamel develops from an extracellular matrix chiefly comprised of amelogenins formed by splicing of 7 (human) or 9 (rodent) exons secreted from specialized epithelial cells known as ameloblasts. Here we examined the role of the 59 amino acid alternatively spliced amelogenin known as leucine rich amelogenin peptide (LRAP) on enamel formation, using transgenic murine models in which LRAP overexpression is driven by an amelogenin promoter (TgLRAP). Beginning in the secretory stage of mouse amelogenesis, we found a reduced thickness of enamel matrix and a loss of Tomes' processes, followed by upregulated amelogenin mRNA expression, inhibited amelogenin secretion and loss of cell polarity. In the presecretory stage (P0) amelogenin m180 mRNA expression was increased 58 fold along with a 203 fold in- crease in MMP-20 expression and 3.5 and 3.2 fold increased in respectively enamelin and ameloblastin. When LRAP was overexpressed on an amelogenin knockout mouse model, the ameloblasts were not affected. Further, expression of the global chromatin organizer and transcription factor SATB1 was reduced in secretory stage TgLRAP ameloblasts. These findings identify a cellular role for LRAP in enamel formation that is not directly related to directing enamel crystal formation as is reported to be the primary

function of full length amelogenins. The effect of LRAP overexpression in upregulating amelogenins, MMP-20 and SATB1, suggests a role in protein regulation critical to ameloblast secretion and matrix processing, to form a mineralized enamel matrix.

Introduction

Dental enamel is formed by mineralization of a protein matrix, which is subsequently removed to allow final mineralization of intercrystalline spaces, resulting in the hardest mineralized tissue in the body. This initially secreted protein matrix is made up primarily of amelogenins, which are alternatively spliced proline rich hydro- phobic proteins and which comprise approximately 90% of all proteins secreted by the enamel forming ameloblast cells (Termine et al., 1980; Simmer, 1995). As these proteins are cleaved and degraded, mineral deposition in the form of hydroxyapatite crystals occurs in a well-ordered pattern (Wen et al., 2001). Therefore, amelogenins appear to have a major role in enamel biomineralization as they function to regulate the orientation, shape and length of enamel hyroxyapatite crystals (Fincham and Moradian-Oldak, 1993; Fincham et al., 1994). In fact, mice lacking the amelogenin gene form only thin mineralized enamel like matrix, lacking the prismatic structure of normal enamel (Gibson et al., 2001).

The primary RNA transcript of amelogenin can be alternatively spliced to form at

least 16 mRNAs, which translate into proteins varying in abundance with proportions that change during the stage of tooth formation (Simmer et al., 1994; Li et al., 1995; Hu et al., 1997; Li et al., 1998). The major expressed amelogenin isoform in the mouse enamel is M180, which is encoded by exons 2–7, skipping exon 4 with exon 1 functioning as a non-coding region (Gruenbaum-Cohen et al., 2009). Despite the quite well known variance of amelogenin proteins, the exact function of each one of them in enamel formation is not fully understood yet.

One of the alternatively spliced amelogenins known as leucine rich amelogenin peptide, LRAP, was first purified from secretory enamel matrix by Fincham et al. (1981) and was later identified by Vies and colleagues as the factor responsible for dentin matrix protein induced osteogenesis (Nebgen et al., 1999). The function of LRAP in dental enamel formation remains elusive. LRAP and full length amelogenin (M180) share many common characteristics in their sequence. The 59 amino acid murine LRAP (M59) contains of the first 33 N-terminus and the last 26 C-terminus amino acids from the full length amelogenin (Gibson et al., 1991). Previous studies of an LRAP overexpressing transgenic mouse generated by Gibson and co-workers showed enamel rods characteristic of normal enamel structure. Furthermore, they found that the LRAP transgene over expressed in amelogenin null mice did not rescue the lack of enamel matrix formation observed in amelogenin null mice, suggesting that LRAP does not have a direct structural role in enamel mineralization (Chen et al., 2003; Habelitz et al., 2006; Gibson et al., 2009).

Cell signaling activities of LRAP in enamel organ epithelial cells was shown by Le et al. (2007) who used human ameloblast lineage cells to show that exogenous LRAP increased amelogenin protein synthesis and reduced notch protein. Addition of exogenous LRAP to mouse enamel organ derived LS8 cells showed co-localization with LRAP (referred to as A-4) and lysosomal-associated membrane protein 1 (LAMP1), as well as an activation of the nitric oxide signaling pathway (Le et al., 2007; Iacob and Veis, 2008).

In this study, we used the TgLRAP mouse model generated by Gibson and co-worker (Chen et al., 2003), which overexpresses LRAP using a bovine amelogenin promoter, to address questions of the role of LRAP in tooth enamel formation. We analyzed developing molars of mice at postnatal days 0, 2, 5, 8 and 10, spanning pre-secretory (day0) to maturation stage ameloblasts (day 10), to determine stage specific effects of LRAP on ameloblast function and enamel formation.

Results

TgLRAP mice produced a distinct phenotype not found in mice overexpressing full length amelogenin (M180), amelogenin null, or LRAP transgene expressed on amelogenin null mice (TgLRAP/amelogenin null)

MicroCT analysis showed a lack of enamel matrix in molars from TgLRAP mice as compared to WT mice with a possible reduction in mineralization of enamel and overlying alveolar bone (Fig. 1). Postnatal day 5 (P5) WT mice showed the presence of a secreted enamel matrix, and ameloblasts demonstrated the elongated polarized appearance typical of secretory ameloblasts with Tomes' processes present (Fig. 2A&B). M180 overexpression (TgM180) did not cause a morphological change in ameloblasts and enamel matrix compared to WT (Fig. 2C&D). In contrast, P5 TgLRAP mice had a dramatic reduction in secretion of enamel matrix with a loss of Tomes' processes. The alignment of TgLRAP ameloblasts was disorganized, and increased in the late secretory cells found over the developing cusp slopes (Fig. 2E&F). Enamel matrix was minimally present in TgLRAP, amelogenin null (Fig. 2G&H) and TgLRAP/ amelogenin null (Fig. 2I&J) mouse molars. However, in contrast to the disrupted ameloblast phenotype in the TgLRAP mouse, ameloblasts in both the amelogenin null and TgLRAP/amelogenin null mice lacked Tomes' processes, but otherwise appeared unaffected (Fig. 2G–J).

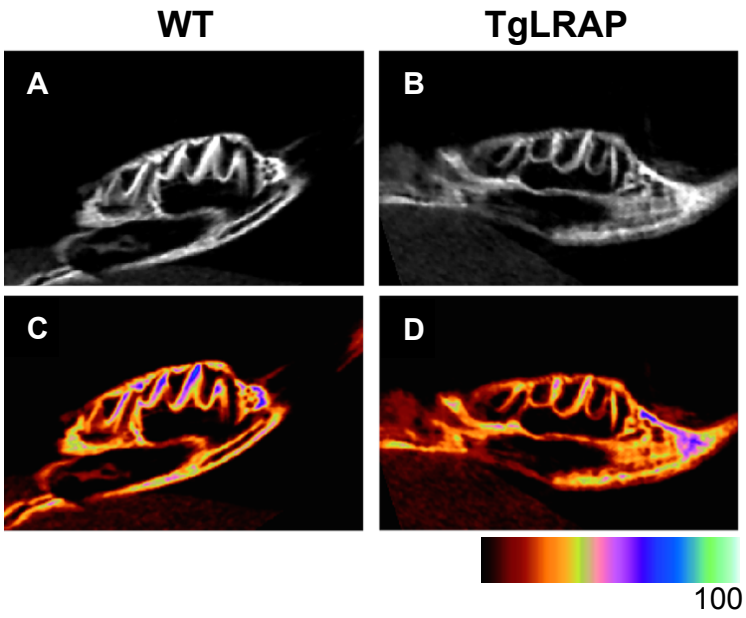


Fig. 1. Micro-CT images of representative first molars of WT and TgLRAP P5 mice. Sagittal views of mandibular first molar from P5 WT (A and C) and TgLRAP (B and D) show similar morphologies. WT (A) molars had a thicker enamel layer as compared to TgLRAP (B) (seen as bright white layer) on the original images. (C and D) Images converted from the original data to color range further confirmed reduced enamel thickness and a decrease in mineralization of enamel in the TgLRAP molar. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

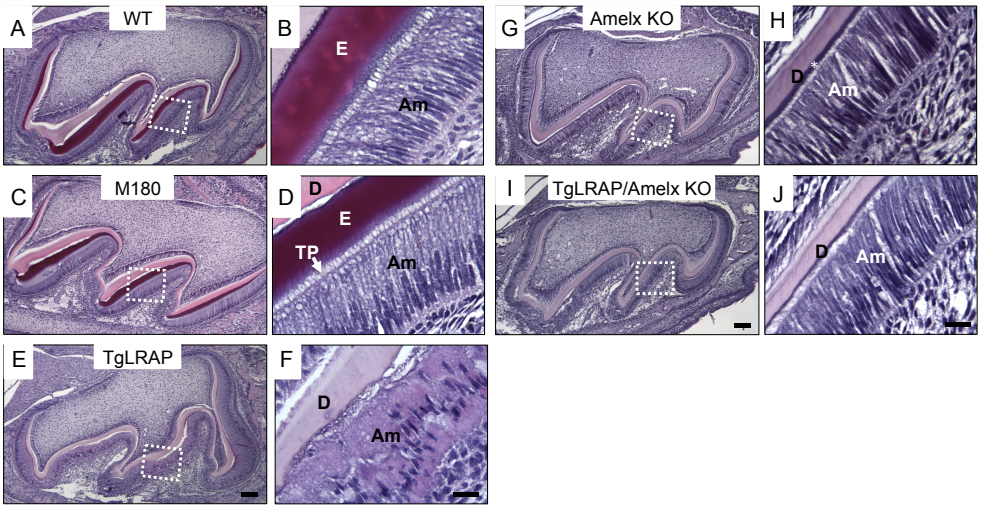


Fig. 2. Hematoxylin and eosin (H&E) staining of 5 day postnatal (P5) molars from various amelogenin transgenic mouse models. (A and B) Secretory ameloblasts on distal cusp slope in WT molar (a boxed area in A) showed the typical polarized appearance of the long cylindrical ameloblast cells with Tomes' process extensions which have laid down enamel matrix. (C and D) M180 secretory ameloblasts appeared morphologically similar to those of WT. Tomes' process extensions were noted throughout the secretory stage. (E and F) TgLRAP secretory stage ameloblasts showed a disorganized pattern of the ameloblasts with multiple layers of cells and less elongated nuclei. Only a small amount of enamel matrix formed, and surface of dentin had an irregular pattern. (G and H) Amelogenin null molar had very thin enamel like matrix (*) and normal appearing elongated ameloblasts, but lacked Tomes' process extensions. (I and J) Ameloblasts of LRAP transgene overexpression in amelogenin null background did not show morphological differences similar to those in amelogenin null mice. Am, ameloblast; D, dentin; E, enamel; TP, Tomes' process. Scale bars 100 μm for A,C,E; 20 μm for B,D,F; 100 μm for G,I; 20 μm for H,J.

In the earlier pre-secretory stage of the molar, ameloblast morphology was not obviously altered in TgLRAP as compared to WT mice (Fig. 3A&B). At this earlier developmental stage enamel matrix has yet to be deposited and only a thin layer of dentin matrix was present in both the WT and TgLRAP tissues. In the secretory stage the WT ameloblasts (Fig. 3C) had elongated compared to pre-secretory cells and were well polarized with Tomes' processes facing the enamel matrix at their apical end. In contrast, the TgLRAP ameloblasts layer (Fig. 3D) was less organized and only a minimal amount of enamel matrix was secreted. Tomes' processes were not detectable in the TgLRAP cells. In the maturation stage of development WT ameloblasts (Fig. 3E) had transitioned to a shortened cell that was still well polarized with a widened enamel space left by the demineralized enamel matrix. The TgLRAP ameloblasts (Fig. 3F) had also transitioned to a shortened cell phenotype in the maturation stage, but remained disorganized in appearance. The lack of an enamel space suggests that only a thin layer of enamel was formed.

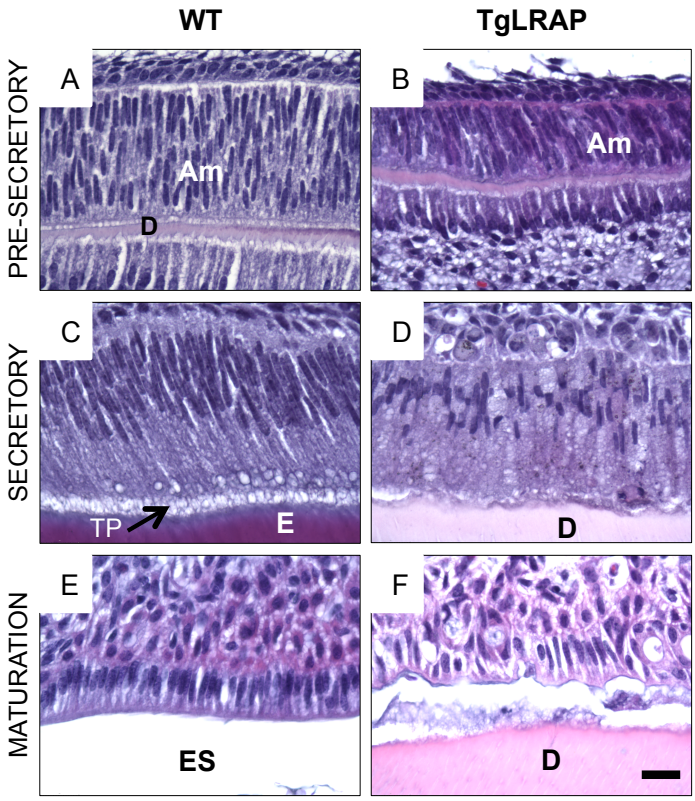


Fig. 3. Stage specific phenotype of WT and TgLRAP ameloblasts. (A and B) At the pre-secretory stage, both WT (A) and TgLRAP (B) ameloblasts were not polarized and appeared similar. At this stage enamel matrix had yet to be deposited and only a thin layer of dentin matrix was present. (C and D) At the secretory stage the WT ameloblasts (C) had elongated and were well polarized with Tomes' process facing the enamel matrix at their apical end. In contrast, the TgLRAP ameloblasts layer (D) was less organized and only a minimal amount of enamel matrix was secreted. Tomes' processes were not detectable in the TgLRAP cells. (E and F) In WT mice maturation stage ameloblasts (E) had transitioned to a shortened cell that was still well polarized with a widened enamel space left by the demineralized enamel matrix. The TgLRAP ameloblasts (F) had also transitioned to a shortened cell phenotype, but remained disorganized appearance. The lack of an enamel space suggests that only a thin layer of enamel was formed. Am, ameloblast; E, enamel; ES, enamel space; D, dentin; TP, Tomes' process. Scale bar 20 μm.

Multiple spliced variants of amelogenin and other enamel matrix proteins are upregulated in P0 TgLRAP molars, but not in P5 TgLRAP molars, as compared to WT molars

As enamel matrix formation was altered in molars of TgLRAP mice, two primer sets based on specific spliced variants of amelogenin mRNA were designed for RT-PCR, to compare the expression profile of amelogenin mRNAs (Fig. 4A). LRAP lacks exon 4 and the majority of exon 6 (6 a, b, and c), and inclusion of exons 8 and 9, occurs when exon 7 is sliced out, ending with the alternative stop codons in exon 9 (Li et al., 1998). RT-PCR showed that the TgLRAP mice had an upregulation of both M180 (as 540 bp) and LRAP (as 180 bp) mRNA in P0 pup molars as compared to WT mouse molars (Fig. 4B). The larger variant ending in exon 7 (Fig. 4B lane 2) was then sequenced to confirm that indeed amelogenin mRNA corresponding to the M180 variant including the full coding region from exons 2 through 7, but excluding exon 4, was upregulated. At P5, which contains primarily secretory stage ameloblasts, there was no obvious difference in expression of LRAP (180 bp) and M180 (540 bp) mRNA between WT and TgLRAP molars. (Fig. 4B lanes 3&4).

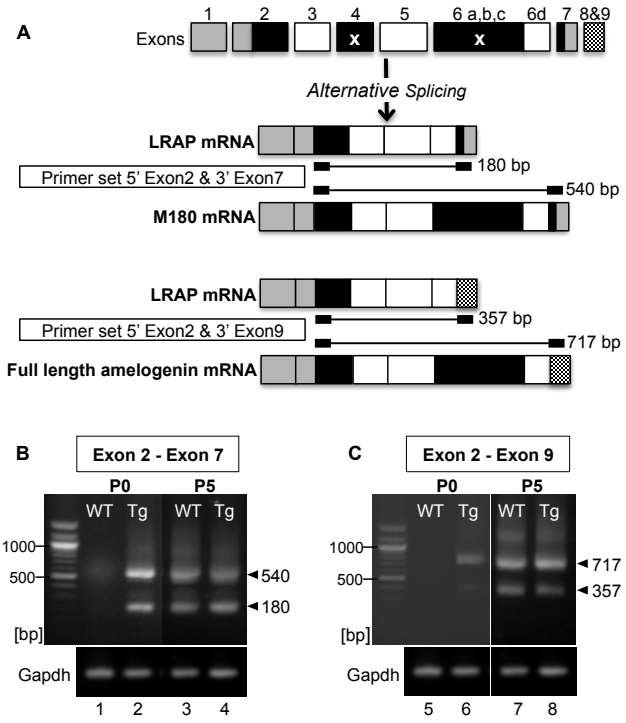


Fig. 4. Expression of multiple spliced variants of amelogenin was upregulated in molars from TgLRAP mice at P0. (A) Spliced variants of amelogenin mRNA and PCR products size using two different primer sets are shown. LRAP lacks exon 4 and the majority of exon 6 (6 a, b, and c) as indicated by x. Inclusion of exons 8 and 9, occurs when exon 7 is sliced out, ending with the alternative stop codons in exon 9 (LI et al.). (B) In P0 WT mice, M180 mRNA expression was barely detected (lane 1), whereas in P0 TgLRAP mice both LRAP (180 bp) and M180 (540 bp) variants were clearly upregulated compared to WT (lane 2). At P5, both splice variants were observed in WT and TgLRAP with a similar relative intensity (lanes 3 and 4). Sequencing results confirmed the dominant upper band from lane 2 matched the M180 amelogenin. (C) A primer set (5′ exon 2 and 3′ exon 9) allowed detection of endogenous amelogenin mRNA but not the LRAP transgene. At P0, RT-PCR products for full length amelogenin (717 bp) and LRAP 8,9 (357 bp) were observed only in TgLRAP mice (lane 6) but not WT (lane 5). At P5, both WT and

TgLRAP mice showed two spliced variants at similar level (lanes 7 and 8).

To confirm the upregulation of amelogenin mRNA expression, quantitative PCR (qPCR) was further performed and it showed a 58 fold upregulation of M180 in P0 TgLRAP tooth buds compared to WT (Table 1). At P5 qPCR did not show a significant difference in M180 expression (data not shown). Interestingly MMP-20, a primary amelogenin degradation enzyme (Lu et al., 2008), was dramatically upregulated (over 200 fold). Among the major enamel matrix proteins besides amelogenin (Uchida et al., 1991; Lee et al., 2003; Iwasaki et al., 2005), both enamelin and ameloblastin were upregulated approximately 3 fold, based on quantitative PCR whereas amelotin expression was relatively unchanged (Table 1).

Table 1: qPCR fold change P0 TgLRAP vs. P0 WT.

Real Time PCR fold change P0 TgLRAP vs. P0 WT		
Gene		Fold Change
M180	⇑	58**
ENAMELIN	⇑	3.5*
AMELOBLASTIN	⇑	3.2*
MMP20	⇑	203**
AMELOTIN	—	1.3

* p-value<0.05, **p-value<0.001

To assess if the TgLRAP mouse had a true upregulation of both the (endogenous/murine) LRAP and full length isoforms, rather than just an increase in the transgene, primers were then designed to include exons 8–9 at the 3′ end which were not included in the transgenic LRAP sequence. At P0 in TgLRAP molars, both full length (717 bp) and (endogenous/murine) LRAP 8,9 (357 bp) variants were upregulated as compared to WT molars when exons 8–9 was included in the primer sequences (Fig. 4C lanes 4&5). Similar to amplification of exons 2–7 (Fig. 4A), both full length and LRAP 8,9 variants were expressed at similar level in P5 WT and TgLRAP molars (Fig. 4C lanes 6&7).

In situ hybridization showed that LRAP was overexpressed in both ameloblasts and odontoblasts at P0

The mRNA analyzed in the TgLRAP was extracted from the whole tooth bud. To further analyze the spatial expression pattern of amelogenin mRNA in ameloblasts and odontoblasts in P0 molars, we completed *in situ* hybridization against both the bovine LRAP transgene and mouse amelogenin. In P0 molars, the LRAP transgene was first evident in the pre-odontoblasts adjacent to the pre-ameloblasts (Fig. 5B). Transgene expression in the ameloblasts was not detected in the pre-ameloblasts but detected only in the more differentiated pre-secretory ameloblasts located along the tooth cusps at P0. Probes specific to full length amelogenin demonstrated a markedly increased expression of

amelogenin mRNA in pre-odontoblasts of the TgLRAP tooth organ in addition to a greater expression of those in pre-ameloblasts and pre-secretory ameloblasts com- paring to WT (Fig. 5C–F). The appearance of LRAP transgene mRNA in the mesenchyme before the ameloblasts, suggests that LRAP in the mesenchyme may have a role in driving the differentiation of ameloblasts with a corresponding increase in amelogenin expression in TgLRAP molar.

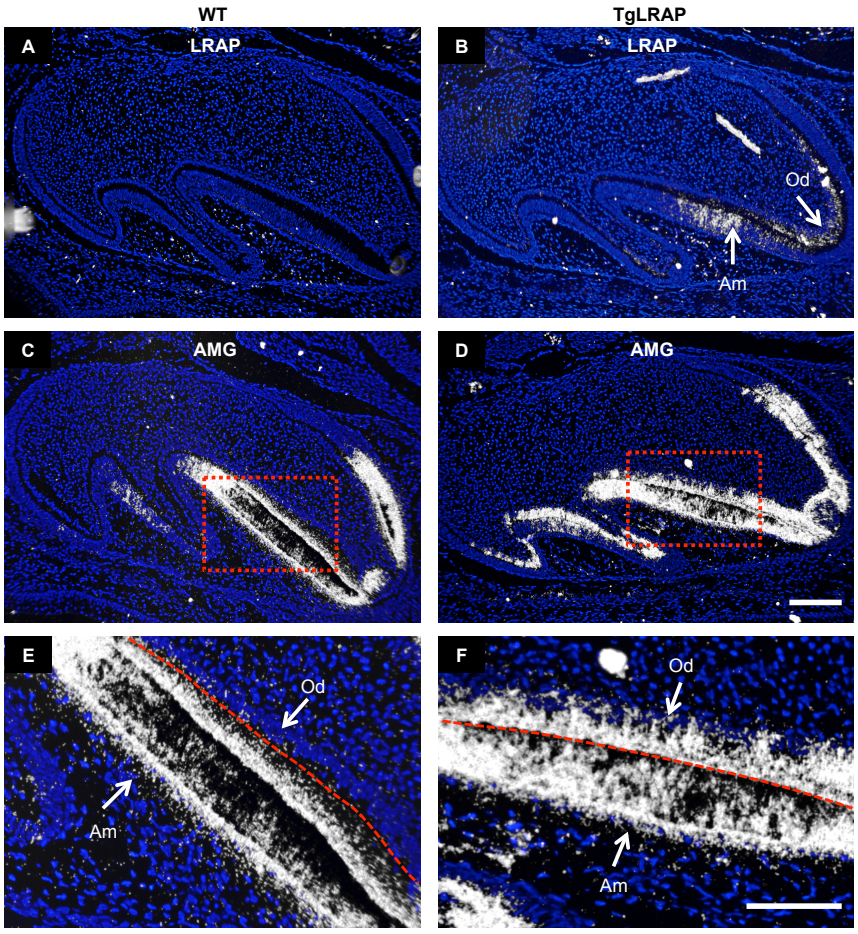


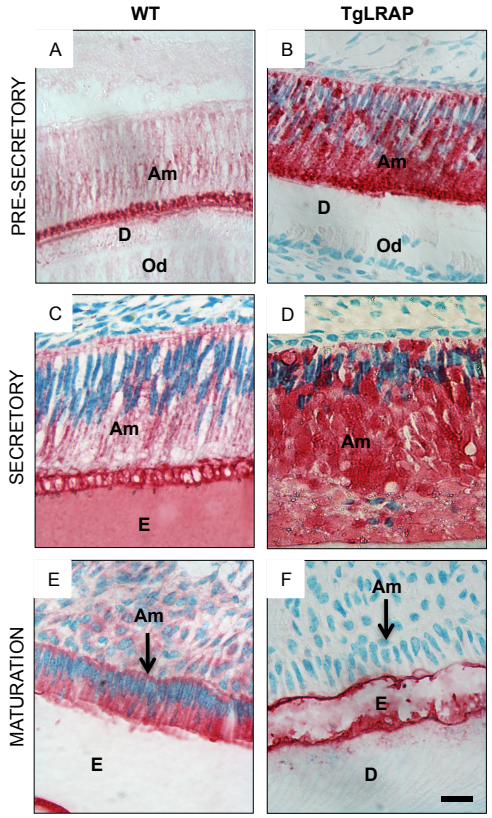
Fig. 5. Spatial expression pattern of amelogenin mRNA by in situ hybridization (ISH) in P0 molars. (A) LRAP mRNA was not detected in WT tissue. (B) In the TgLRAP mice the LRAP transgene probably as well as endogenous LRAP mRNA was localized in the odontoblasts adjacent to pre-ameloblasts, and in the pre-secretory ameloblasts. (C and E) ISH with a full-length mouse amelogenin (AMG) probe showed abundant expression in pre-ameloblasts and pre-secretory ameloblasts in WT molar. Higher magnification of boxed area (E) showed amelogenin mRNA was detected only in the pre-secretory ameloblasts with the red dashed line indicating the future enamel–dentin junction between the ameloblasts and odontoblasts which have yet to form matrix. (D and F) In the TgLRAP mice AMG mRNA was abundantly expressed in pre-ameloblasts and pre-secretory ameloblasts, as well as in adjacent odontoblasts. Am, ameloblast; Od, odontoblast. Scale bars 100 μm.

TgLRAP mice showed increased amelogenin protein in pre-secretory and secretory ameloblasts with a loss of regulation of amelogenin secretion

To further examine if amelogenin protein synthesis or secretion was altered, amelogenin was immunolocalized in P2 (pre-secretory), P5 (late secretory) and P10 (early maturation) molars. Amelogenin (AMG) immunolocalization showed that presecretory ameloblasts found in P2 molars of TgLRAP mice had increased amounts of amelogenin protein as compared to WT mice, but no obvious alteration of ameloblast morphology (Fig. 6A&B). Secretory ameloblasts found in P5 WT molars showed elongated shape and began to secrete amelogenin proteins into the extracellular enamel matrix (Fig. 6C). However P5 TgLRAP mouse molars showed less elongated and disorganized nuclei and an apparent

lack of continued secretion of amelogenin protein into the extracellular enamel space (Fig. 6D). At P10, WT ameloblasts were maturation stage with shorter the height, but still polarized location of nucleus, while in contrast the TgLRAP mouse ameloblasts layer was less organized and showed a loss of polarization as compared to WT (Fig. 6E&F). Amelogenin protein could be detected in the WT maturation stage ameloblasts, possibly due to re-uptake of hydrolyzed amelogenin fragments from the matrix as reported previously (Reith and Cotty, 1967; Smith, 1979), while the P10 TgLRAP ameloblasts showed no amelogenin positive staining.

Figure 6: Immunohistochemical localization of amelogenin protein on molar ameloblasts.



(A) Presecretory ameloblasts in P2 WT mice showed amelogenin protein mainly localized on the apical region of cytoplasm. (B) Besides the strong immunoreaction on the apical region, entire cytoplasm of presecretory stage ameloblasts in P2 TgLRAP mice showed highly intense amelogenin protein positive reaction. (C) Amelogenin protein was detected on entire cytoplasm and Tomes' process of secretory stage ameloblast of P5 WT mice. (D) Secretory stage ameloblasts of P5 TgLRAP mice formed a disorganized layer and demonstrated amelogenin protein highly retained in the cytoplasm. (E) At maturation stage, ameloblasts of P10 WT mice were amelogenin immunopositive. (F) However ameloblasts in P10 TgLRAP mouse molars lacked

polarity and were amelogenin immunonegative. Am, ameloblast; E, enamel; Od, odontoblast; D, dentin. Scale bar, 20μm.

Apoptosis was increased in the late secretory stage ameloblasts of TgLRAP mouse molars

As apoptosis is found in transition and maturation stage ameloblasts a TUNEL assay was performed to determine if stage specific patterns of apoptosis differed between WT and TgLRAP tissues. The late secretory ameloblasts found on the distal slope of the mesial cusp of the P5 mouse molar showed TUNEL positive staining, which was not present in the WT mouse ameloblasts (Fig. 7A&B). This area is the location where cells dramatically lose organized layer and showed the greatest accumulation of amelogenin protein in the cytoplasm. The transitional stage ameloblasts present at P8 in the WT mouse molar showed positive TUNEL stain, but no TUNEL stain was present in P8 TgLRAP molars (Fig. 7C&D). The evidence of earlier apoptosis in ameloblasts from TgLRAP molars as compared WT mice, suggests earlier timing of ameloblast differentiation in TgLRAP mouse molars.

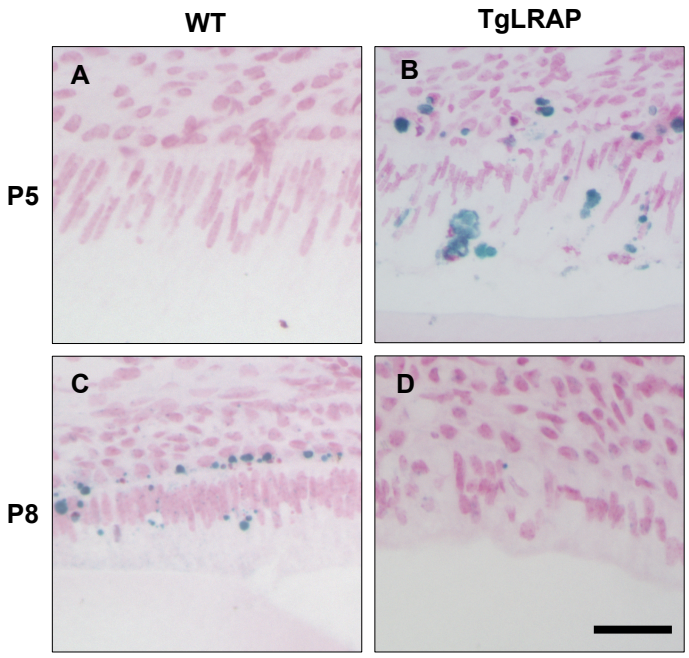


Fig. 7. TUNEL staining on P5 and P8 molar ameloblasts. (A) P5 WT molars had no TUNEL positive ameloblasts. (B) P5 TgLRAP molar showed TUNEL positive staining (in blue) in the region corresponding to the most differentiated secretory stage ameloblasts (C) AT P8 WT molars had post-secretory stage ameloblasts where TUNEL positive staining was observed. (D) Ameloblasts of P8 TgLRAP mice showed post-secretory morphology and slight TUNEL positive staining was observed. Scale bar 30 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Expression of the tissue specific gene expression regulator, special adenine and thymine-rich sequence binding protein 1 (SATB1), was decreased in TgLRAP mice ameloblasts

SATB1, a global chromatin organizer and transcription factor in progenitor cells (Cai et al., 2006; Pavan Kumar et al., 2006), was observed in the nucleus of pre-secretory and secretory stage ameloblasts. Pre-secretory WT and TgLRAP ameloblasts expressed the highest levels of SATB1 (Fig. 8A&B). Early secretory and pre-secretory WT ameloblasts showed similar levels of SATB1 nuclear staining, and SATB1 was dramatically reduced at the late secretory stage (Fig. 8A,C,E). In contrast, TgLRAP mouse molars showed a dramatic reduction in SATB1 immunostaining in early secretory stage ameloblasts to levels similar to those found in late stage WT molars (Fig. 8D&E), and was further reduced in the late secretory TgLRAP ameloblasts below the levels found in WT molar ameloblasts (Fig. 8E&F).

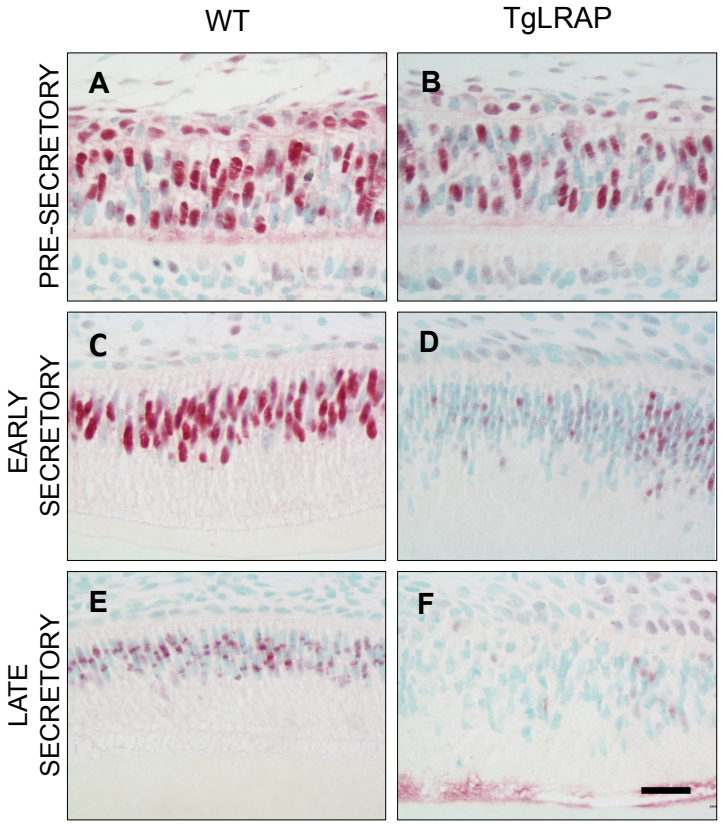


Fig. 8. Expression of the gene regulation protein SATB1 was decreased in TgLRAP ameloblasts. (A and B) At the pre-secretory stage, ameloblasts as well as stratum intermedium cells of both WT (A) and TgLRAP (B) mice strongly expressed SATB1 protein in the nucleus. (C and D) In the early secretory stage, SATB1 expression in the WT ameloblasts (C) remained strong in the nucleus similar to pre-secretory stage, but there was a marked decrease in the TgLRAP ameloblasts (D). (E and F) In the differentiated late secretory ameloblasts, SATB1 expression was reduced in the WT ameloblasts (E) compared to its earlier stage, and the corresponding stage of TgLRAP ameloblasts (F) demonstrated very weak expression. Scale bar, 20 μm.

Discussion

TgLRAP mice were generated by Gibson and co-workers (Chen et al., 2003) as a part of a larger question to determine whether LRAP could rescue the amelogenin null phenotype where enamel matrix formation is inhibited. In studies using this mouse model, they reported that the molars of TgLRAP mice had a normal enamel structure, but with a slightly irregular enamel surface characterized by pitting (Chen et al., 2003). Our histological analyses and microXCT showed that in addition to an irregular enamel surface, the entire enamel layer of molar was thin, suggesting a defect in matrix protein secretion. We then compared the enamel of TgM180 mice to that of the TgLRAP, and found that unlike TgLRAP, the TgM180 mouse molars formed a matrix with a thickness similar to WT mice, no obvious changes in ameloblast phenotype.

The difference between LRAP and M180 amelogenin is that the M180 transcript contains exon 6 a,b,c (540 base pair), which codes for a large central hydrophobic amino acid sequence in the synthesized protein (Gibson et al., 1991). Neither LRAP nor M180 contains exon 4 or exons 8 and 9. The disruption in enamel formation when LRAP is overexpressed, suggests a specific effect of LRAP on ameloblast mediated amelogenesis.

When we compared the ameloblasts of TgLRAP mouse molars to WT mouse molars, these changes appeared to be stage specific. The pre-secretory

ameloblasts found in P0 and P2 mouse molars appeared similar in the TgLRAP and WT mice, even though in the TgLRAP molars both full length amelogenin mRNA and protein expression was upregulated. Early secretory stage ameloblasts located on the outer cusp surfaces also appeared relatively normal, and the first obvious signs of an abnormal ameloblast phenotype occurred in late secretory ameloblasts on the distal slope of the mesial cusp of P5 molars. These ameloblasts appeared to show less elongated nuclei, form disorganized layer, and have increased apoptosis. Further differentiation of ameloblasts at the maturation stage (P10 molars) was completely disrupted and the cells overlying the thin enamel matrix appeared as disorganized epithelial cells. This is the first report of such a significant effect of LRAP overexpression on ameloblast function and differentiation.

This phenotype, showing loss of organized cell layers and protein retention was even more striking in that it only occurred in the TgLRAP mice, in which the LRAP transgene was overexpressed on a wild type background. When LRAP was overexpressed in the amelogenin null background, consistent with reports of the amelogenin null mice, ameloblasts did not have Tomes' processes, but appeared otherwise largely unaffected. Also consistent with previous reports, we found that the amelogenin null mice secreted a poorly mineralized thin layer of enamel (Gibson et al., 2001), and we found that this enamel matrix morphology was not altered by the LRAP overexpression.

This observation, led us to further investigate the effect of LRAP overexpression on amelogenin synthesis, as well mRNA expression of other enamel matrix proteins and proteinases. Immunohistochemical staining of TgLRAP mice showed that amelogenin protein synthesis was increased in pre-secretory ameloblasts as compared to WT mice. This was consistent with *in situ* hybridization, which also showed both LRAP and amelogenin upregulated in both ameloblasts as well as odontoblasts. These results suggest a specific effect on LRAP in upregulating amelogenin protein synthesis that is not cell specific.

To further investigate the effects of stage specific upregulation of LRAP in ameloblasts, we completed PCR analysis of amelogenin mRNA synthesis in molars containing primarily pre-secretory ameloblasts (P0) and molars containing primarily secretory stage ameloblasts (P5). PCR showed an upregulation of both LRAP and M180 amelogenin splice variants in P0 mouse molars, with no further upregulation of amelogenin mRNA in P5 TgLRAP as compared to WT mouse molars, suggesting a specific role of transgene LRAP in the early stage of ameloblast differentiation.

To determine whether native LRAP was upregulated as opposed to increased expression of the transgene, we then amplified PCR products ending with the alternate C terminus coded by exons 8 and 9 (Li et al., 1998), as these exons were not included in the transgene LRAP. This PCR analysis showed that the larger amelogenin splice variant containing all exons with the exception of exon

4, was specifically a retention of amelogenin, as occurs in an amelogenin P70T mutation, also results in an upregulation of MMP-20 mRNA. Therefore, it seems that MMP-20 expression may be co-regulated with amelogenin at the secretory stage of amelogenesis.

We found that increased amelogenin expression during the pre-secretory stage (P0) did not result in an effect on ameloblast morphology. In TgLRAP mouse molars, amelogenin secretion was limited to the early secretory stage. At the late secretory stage, ameloblasts did not appear to secrete amelogenin, and their morphology was dramatically affected with a less elongated nuclei and disorganized cell layer. Previous *in vitro* studies by Tompkins et al. (2005) of tooth organs grown in culture, found that addition of exogenous LRAP (A-4) to the culture medium, inhibited pre-ameloblast polarization. Our *in vivo* studies suggest that when LRAP is present intracellularly in pre-secretory ameloblasts, polarization is not affected; possibly indicating that an effect of LRAP on ameloblasts is related to an overexpression of LRAP secreted protein. In support of this possibility is the finding that when LRAP was over expressed on an amelogenin null background mice, no phenotypic changes were observed.

When only M180 was overexpressed we found no ameloblast phenotype, further indicating the unique function of secreted LRAP in altering ameloblast morphology. Ravindranath et al. (2007) showed that addition of exogenous LRAP protein to E16 molar explants decreased ameloblast height, suggesting

LRAP may directly enhance differentiation and promote early ameloblast maturation. We found that the non-amelogenin matrix proteins, enamelin and ameloblastin, also exhibited increased early gene expression in the TgLRAP mice, further supporting the possibility that LRAP promotes earlier ameloblast differentiation.

As apoptosis is only present in transitional and maturation stage ameloblasts (Smith and Warshawsky, 1977) our finding of a positive TUNEL stain at P5 suggests that the TgLRAP ameloblasts may have differentiated toward transitional stage ameloblasts. Our findings demonstrate an increase in cell death specifically in the late secretory stage ameloblasts at P5. At this age most ameloblasts in the rodent molar would be expected to be secretory stage cells, however, the potential increase in apoptosis suggests that transition stage related apoptosis occurred at an earlier time point. Indeed at P8 TUNEL positive reactions were observed at transitional stage ameloblasts in WT mouse molars, while the TgLRAP P8 ameloblasts did not show any TUNEL positive reactions.

If LRAP modulates ameloblast differentiation, it seems that LRAP overexpression may trigger a more overarching mechanism controlling ameloblast differentiation. Recent studies in our laboratory have pointed to the role of special AT sequence binding protein (SATB1) in ameloblast differentiation. SATB1 is known to regulate large-scale chromatin remodeling in certain cell types and progenitor cells (Cai et al., 2003, 2006; Kumar et al., 2007; Han et al., 2008; Agrelo et al.,

2009). In epidermal tissue, SATB1 functions as one of the direct downstream components for the global regulatory network of p63 and contributes in epidermal tissue morphogenesis by establishing tissue-specific chromatin organization and gene expression in progenitor cells (Han et al., 2008; Fessing et al., 2011). Epidermis of SATB1 null mice show impaired morphology and downregulation of cell proliferation (Fessing et al., 2011). We found that ameloblasts of the SATB1 null mouse lack Tomes' processes and have an earlier transformation into maturation stage ameloblasts (unpublished data, Zhang et al.). Downregulation of SATB1 in the LRAP overexpressor mice, suggests the possibility that interactions between LRAP protein and SATB1 protein may modulate ameloblast differentiation.

Along with the altered epithelial cell signaling reported here, LRAP has also been reported to alter cellular pathways in mesenchymal cells, including MAPK signaling in cementoblasts (Boabaid et al., 2004) and activation of the WNT canonical pathway in bone marrow stem cells (Warotayanont et al., 2009; Wen et al., 2011). These findings and our report of LRAP effects on ameloblast differentiation, clearly show that this alternatively spliced amelogenin does alter cell signaling. Additional studies are needed to further understand how this small alternatively spliced amelogenin protein regulates gene expression and ameloblast differentiation in tooth formation.

Methods

Animal models

All animal procedures were performed after approval by the University of California San Francisco and University of Pennsylvania IACUC. Amelogenin null, TgLRAP, and TgM180 mice used for these studies were provided by Dr. Carolyn Gibson and as previously described (Gibson et al., 2001; Chen et al., 2003; Gibson et al., 2007) both TgLRAP, and TgM180 mouse models were made by incorporating a plasmid containing the amelogenin promoter with either cDNA encoding for LRAP (TgLRAP) or cDNA containing amelogenin exons 1, 2, 3, 5, 6 and 7 (TgM180). TgLRAP mice in an amelogenin null background were made by mating TgLRAP + males with amelogenin null females. As the murine amelogenin gene is sex linked on the X chromosome this mating resulted in all males being amelogenin null with ~ 50% expressing the LRAP transgene. Wild type (WT) mice were generated as littermates.

Micro-computed tomography (micro-CT)

Level of mineralization of first molar was accessed on non-decalcified hemi-mandibles of 3 samples from P5 WT and TgLRAP respectively by micro-computed tomography (SkyScan1076; Bruker- microCT, Kontich, Belgium) with X-ray source operating settings at 100 kV and 0.1 mA. After reconstitution of images by NRecon software (Bruker-microCT), appropriate sagittal imaging

planes were selected from three orthogonal sections centered at a level containing three buccal cusps inside the reconstructed space using Data Viewer software (Bruker-microCT).

Immunohistochemistry and H&E staining

Maxillae at P0, 2, 5, 8, and 10 were prepared for immunohistochemistry, by immediate immersion in 4% paraformaldehyde (PFA)/ 0.06 M cacodylate buffer (pH 7.3) for 1 day at 4 °C followed by decalcification in 8% EDTA (pH 7.3) at 4 °C for 10 days, The samples were then dehydrated through a graded series of ethanol followed by routine embedding in paraffin and sectioning.

After deparaffinization, the sections of maxillae were incubated with 10% swine and 5% goat sera followed by incubation with rabbit anti-human full length recombinant amelogenin (1:500; Le et al., 2007) dilution or rabbit anti-SATB1 antibody (1:200; Abcam Inc., Cambridge, MA) overnight at room temperature. A biotinylated swine anti-rabbit IgG F(ab′)2 fraction (DakoCytomation Inc., Carpinteria, CA) was used as the secondary antibody for 1 h at room temperature incubation. Following incubation with alkaline phosphatase conjugated streptavidin (Vector Laboratories Inc., Burlingame, CA) for 30 min, immunoreactivity was visualized using a Vector® Red kit (Vector Laboratories Inc.) resulting in pink/red color for positive staining. Counter-staining was performed with methyl green. Negative control was done with nor- mal rabbit sera. Additional sections were utilized for standard hematoxylin and eosin (H&E)

staining after deparaffinization.

PCR analysis

Total mRNA for each sample, maxillary first molars were dissected from a postnatal day 0 and day 5 WT and transgenic mice. Molars from four pups (n = 4) were included for each timepoint. Total RNA was isolated using RNeasy Mini kit (QIAGEN, Germantown, MD). An aliquot containing 1 μg of total RNA was reverse transcribed to cDNA using SuperScript® III Reverse Transcriptase (Invitrogen, Carlsbad, CA) (see Table 2 for list of primers). Polymerase chain reaction amplification for RT-PCR was performed with the Hot Start Taq kit (Qiagen) by first incubating the reaction mixture at 95° for 5 min, followed by 94 °C, 57 °C, and 72 °C for 1 min each for 30 cycles and then 72 °C for 10 min with the same condition used for all primers. The products were visualized on a 2% agarose gel with ethidium bromide staining. Real-time PCR gene expression was characterized by quantitative PCR using the ABI 7500 system (Applied Biosystems, Carlsbad, CA). cDNA was amplified with the Fast Start SYBR Green master mix (Roche. Indianapolis, IN). Relative expression levels of target genes were analyzed by the ΔΔCt method as published previously (Thomsen et al., 2010). All data were analyzed by Student *t*-test by using Prism software (GraphPad Software Inc., San Diego, CA, USA).

In situ hybridization

Maxillae from P0 mice were fixed with 4% PFA/0.06 M cacodylate buffer (pH 7.3)

for 1 day at 4 °C then dehydrated through a graded series of ethanol followed by routine embedding in paraffin and sectioning. Complementary DNAs corresponding to LRAP and full length amelogenin were used to generate antisense and sense riboprobes as described (Albrecht et al., 1997). The transgenic LRAP probe was a 42 bp probe that crossed the boundary between exons 5 and 6d excluding exon 6 domains a,b,c. The amelogenin probe was a 512 bp probe corresponding to the region from exons 2 to 6d of full length amelogenin. The sequences of the probes were confirmed by auto- mated DNA sequencing. The riboprobes were labeled with ^{35}S and *in situ* hybridization was done as previously described (Sundin et al., 1990; Albrecht et al., 1997; Ferguson et al., 1999). Following hybridization and washing emulsion-dipped slides were exposed to beta emissions from the ^{35}SUTP-labeled riboprobes for 4–6 days. The sections were counterstained with a nuclear stain (Hoechst Stain; Sigma Aldrich, St. Louis, MO). Hybridization signals were detected by darkfield optics, and the nuclear stain was visualized by epifluorescence. The images of the signals and counter-stain were superimposed using Adobe Photoshop software (Adobe, San Francisco, CA) to facilitate identification of cells expressing a particular gene.

TUNEL assay

To label and detect DNA strand breaks, *in vitro* terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining was performed on the P5 paraffin embedded molar sections using TACS·XL® (Trevigen Inc.,

Gaithersburg, MD) according to the kit instructions.

Imaging

Histological images were taken with a Nikon Eclipse E3800 micro- scope (Melville, NY) using a digital camera (QImaging Inc., Surrey, Canada) and SimplePCI imaging software version 5.3.1.

Table 2: Primer sequences for Enamel matrix gene expression by RT-PCR or qPCR

Gene	Primer Pair
Amelogenin Exon 2-7	F: 5'GCTATGCCCCTACCACCTC 3' R: 5' GTCCACTTCTTCCCGCTTG 3'
Amelogenin Exon 2-9	F: 5'AACCATCAAGAAATGGGGACC 3' R: 5' ACTACATGCCATTGTGTTCTG 3'
Full length Amelogenin (for qPCR)	F: 5'CAGCAACCAATGATGCCAGTTCCT 3' R: 5' ACTTCTTCCCGCTTGGTCTTGTCT 3'
Ameloblastin	F: 5' CTGTTACCAAAGGCCCTGAA 3' R: 5' GCCATTTGTGAAAGGAGAGC 3'
Amelotin	F: 5' ATCAGCCCAGTCATTACCAAAG 3' R: 5' AGGTCTGACCCCAGAGTGAG 3'
Enamelin	F: 5' GCTTTGGCTCCAATTCAAAA3' R: 5' AGGACTTTCAGTGGGTGT 3'
MMP20	F: 5' CTCGTCCTTTGATGCAGTGA 3' R: 5' TGGACATTAGCTGGGGAAAG 3'
Gapdh	F: 5' GGA CGC ATT GGT CGT CTG G 3' R: 5' TTT GCA CTG GTA CGT GTT GAT 3'

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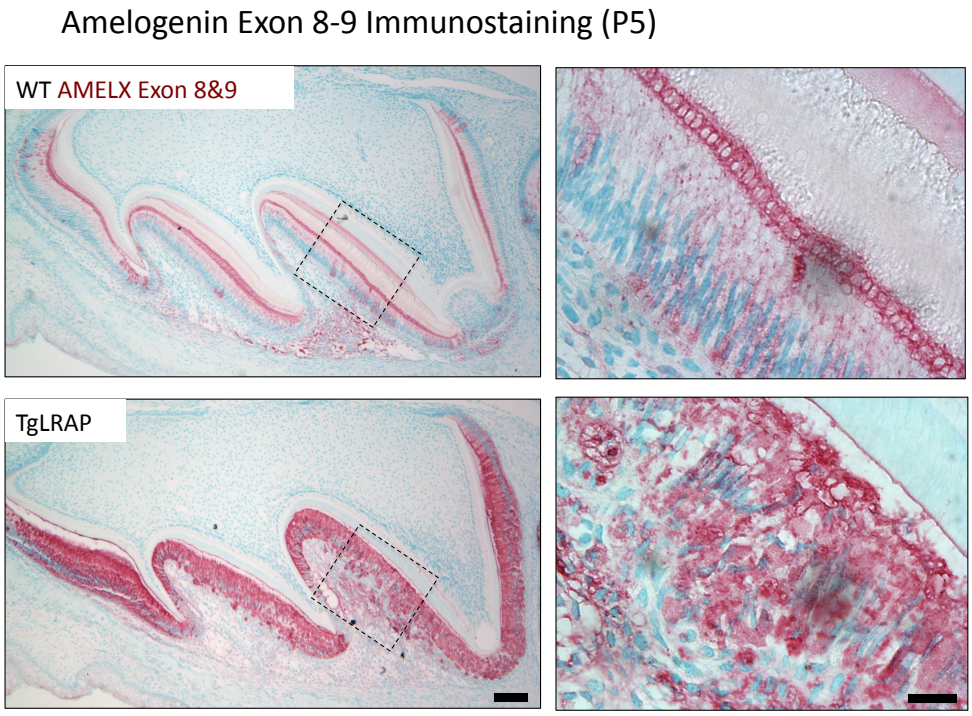
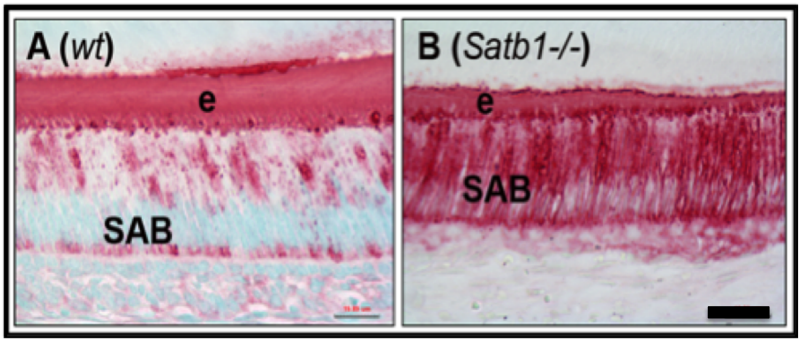


Supplemental 1: Immunodetection of amelogenin exon 8&9 is increased in the secretory ameloblasts of the TgLRAP molar. (A) WT P5 maxillary molar shows expression of exon 8&9 in the cytoplasm of the ameloblasts (B) In the TgLRAP ameloblasts immunodetection of exon 8&9 is increased. (C) The mouse predicted exon 8&9 coded protein sequence. The shaded area represents the sequence of the antibody raised for detection of exons 8&9, which included the last three amino acids of the exon 8's coding sequence. Scale bar 100 µm for A,C; 20 µm for B,D; 100 µm.



Supplemental 2: Amelogenin expression is increased in the SATB1 Null mouse (A) Secretory ameloblasts in a P13 mandibular incisor express amelogenin (red stain). (B) In the Satb1 Null increased retention of amelogenin is present with a thinner enamel matrix.

Appendix 1. TgLRAP Transgene Expression

To generate a mouse that overexpressed the short amelogenin LRAP a construct containing the bovine LRAP sequence was first created. The vector used to generate the TgLRAP Plasmid was pBAXbLRAP-21 and was constructed by combining 5.5 kb of upstream bovine X chromosomal amelogenin genomic sequence, plus exon 1 and intron 1 to exons 2 through 7 of bovine LRAP cDNA, followed by 500 bp of 3' genomic DNA which included the polyadenylation signal sequence and an engineered EcoRI site (Fig 1). The genomic upstream and downstream DNA was taken from cloned segments of the bovine X-chromosomal amelogenin gene (Chen et al., 1998; Gibson et al., 1992), and the cDNA was taken from a cloned LRAP PCR product and from pUCVL2A2 containing full length bovine amelogenin message. The final cloning vector was pSL1180 (Amersham Biosciences, Piscataway, NJ), which was removed by digestion with XhoI prior to microinjection. DNA sequence was verified for all constructs.

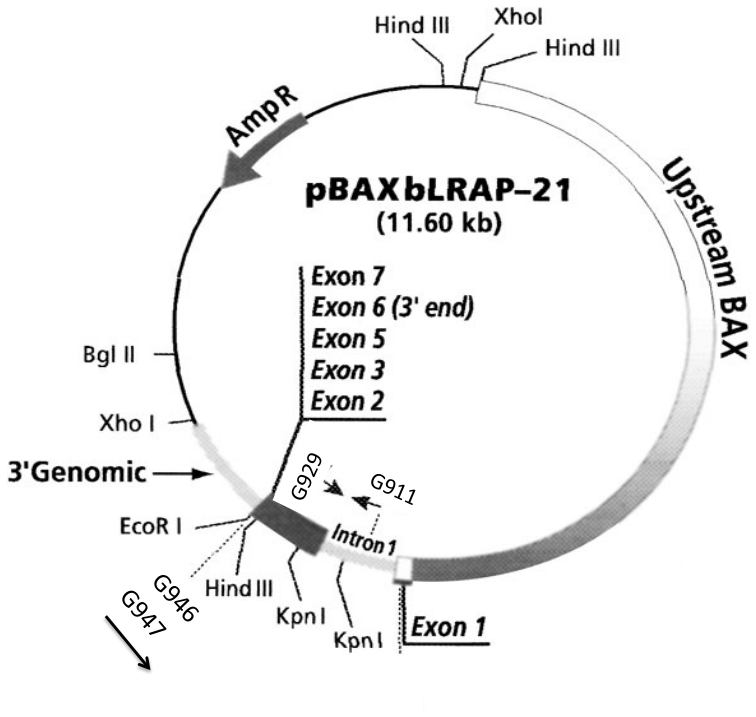


Figure 1. The LRAP expression vector contains the upstream and downstream regions of bovine X-chromosomal amelogenin genomic DNA, including all of exon 1 and intron 1. Also included are exons 2, 3, 5, the 3′ end of 6 and 7 from bovine LRAP cDNA.

Although the endogenous amount of murine LRAP protein normally present is not known, it was shown that bovine LRAP was easily detected on the protein level in the TgLRAP mice. An antibody to bovine LRAP raised in a rabbit host was designed using an eight amino acid peptide located at the junction between exon 5 and 6d and taking advantage of the decreased homology between murine and bovine LRAP in that region with 2 of the 8 peptides differing between the species (Figure 2). In total six amino acids differ between bovine and murine

LRAP. Testing the antibody on a murine secretory stage (P5) WT tissue section failed to produce an immunoreaction, but a clear reaction was seen when the antibody was tested on a TgLRAP tissue section of a similar age (Figure 2). This suggests the antibody is specific to bovine LRAP. Both tissues were immunopositive to a full length amelogenin antibody.

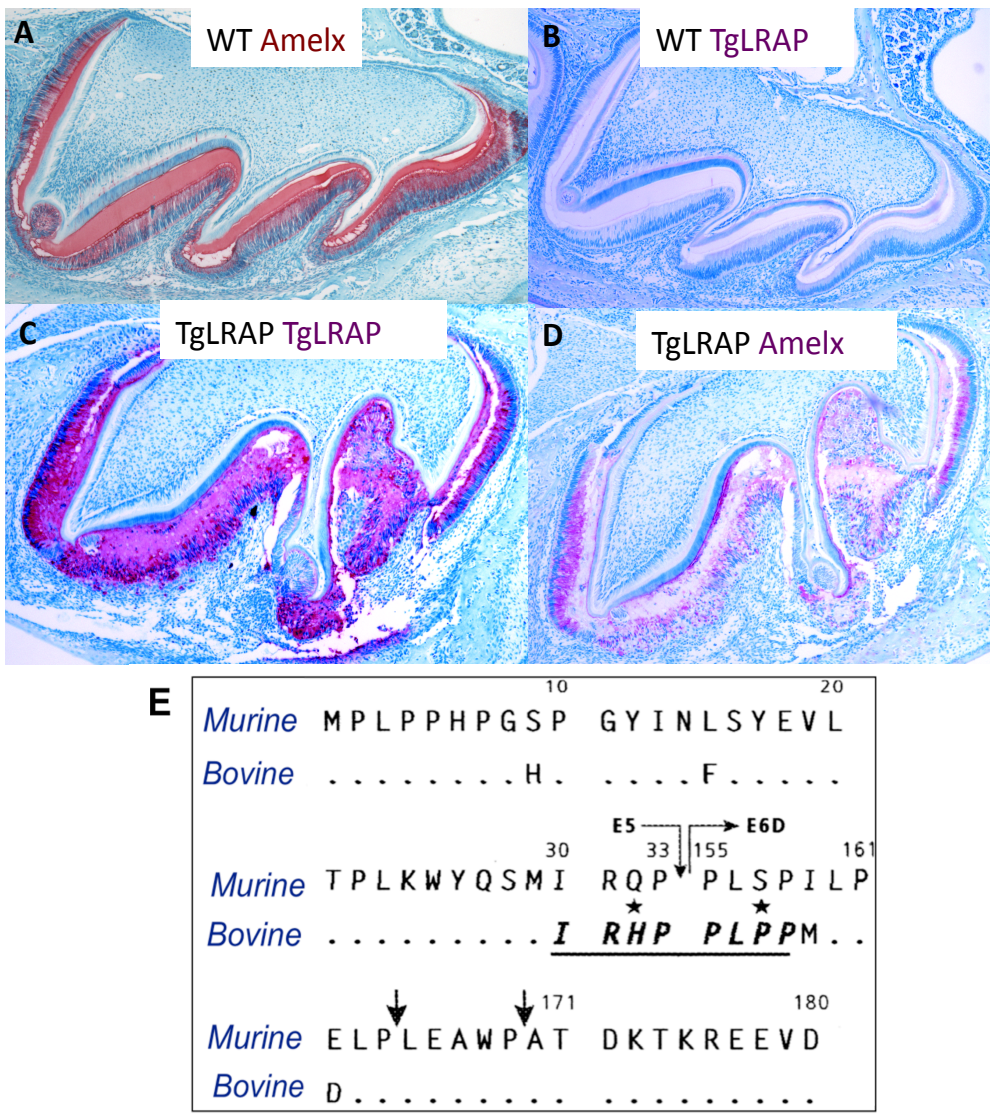


Figure 2. Expression of transgenic LRAP. (A) Immunodetection of amelogenin was seen in the cytoplasm throughout a P5 WT molar. The LRAP transgene could not be detected in a WT section (B) using an antibody specific to bovine LRAP. In the TgLRAP mouse (C) amelogenin is abundantly expressed and (D) LRAP was also clearly visualized, but to a lesser extent than amelogenin. (E) Six differences exist between the bovine and murine LRAP sequence. A polyclonal antibody to bovine LRAP was generated from residue 30 to 33 and 155 to 158 where two of the eight residues between murine and bovine LRAP differed.

Genotyping PCR

The mice were transgene positive if a PCR product from primers G 929 and G 911 is present (Fig 3).

G 929 (bovine intron 1): 5' TAA GTC CTG TCA GTC AGA ACA CCT 3'

G 911 (exon 3): 5' TTG ATA TAA CCA GGG TGC CCA GGA 3'

PCR product = 608 bp

The mice are positive for the LRAP transgene if a product from primers 733 and G946 is present (Fig 3).

733 (exon 2): 5' CAT GGG GAC CTG GAT TTT GTT TG 3'

G946 (exon 7): 5' CTA AGT AAG AAT TCG GTG GTG 3'

PCR product = 332 bp

PCR conditions for the above indicated transgenic mouse strains using the Hot Start Taq Polymerase kit (Qiagen) were as follows, Denature: 95 ^{o}C 5 mins Denature: 94 ^{o}C 1 min Anneal: 60 ^{o}C 2 mins Extend: 72 ^{o}C 3 mins 30 cycles.

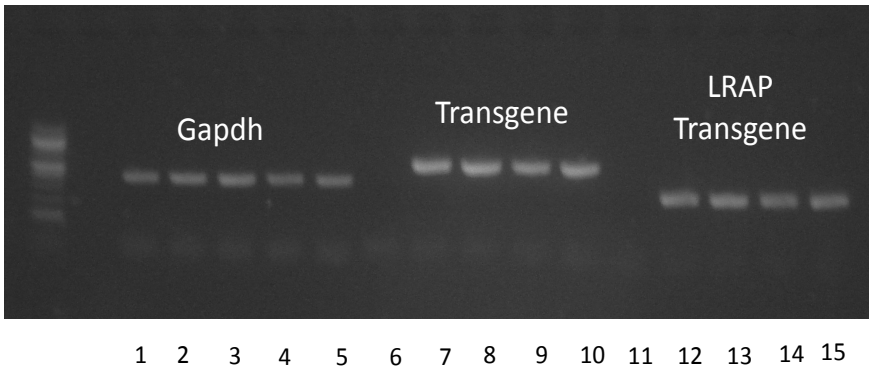


Figure 3: Genotyping of Postnatal day 5 TgLRAP Liter. In this of 5 pups produced from a TgLRAP heterozygous sire and TgLRAP heterozygous dam. A space is present after the ladder and lanes 1-5 are positive for the internal control Gapdh. Lanes 6 & 11 indicate that pup was negative for the transgene using two different primer sets. The other 4 pups pups (lanes 7-10 & 12-15) were positive for the transgene based on the two primer sets.

Partial sequence (including LRAP insert), pCMV5-LRAP19

CGCCCCGTT GACGCAAATG GGCGGTAGGCGTGTACGGTGGGAGGTCTAT ATAAGCAGAG
CTCGTTTAGTGAACCGTCAGAATTCAGATCTGGTACCACGCGTATCGATA
AGCTTGCATGCCTGCAGGTCGACTCTAGA**GGATCC**
ACCATGGGGACCTGGATTTTGTTTGCCTGCCTCCTGGGAGCAGCCTTCTCTATGCCT
CTACCACCTCATCCTGGGCACCCTGGTTATATCAACTTCAGCTATGAG
GTGCTCACCCCTCTGAAGTGGTACCAGAGCATGATAAGACACCCGCCTCTG
CCCCCCATGCTTCCTGACCTGCCTCTGGAAGCTTGGCCAGCAACAGACAAGACCAAGCGG
GAGGAAGTGGATTAA**GGATCC**CGGTGGCATCCCTGTGACCCC…

Key:

CMV promoter

CMV vector

Bam site

LRAP signal sequence

Exon 2 (partial; ATG is beginning of mature protein)

Exon 3 (all)

Exon 5 (all)

Exon 6 (3' end)

Exon 7 (5' end, includes TAA stop codon)

= 180 bp

Appendix 2. Further characterization of the TgLRAP model

Cytokeratin 14

Cytokeratins are intermediate filaments that serve as cytoskeletal proteins produced exclusively by epithelial cells and consist of a highly conserved family of about 20 polypeptides, distinguished from each other by molecular weight and immunoreactivity (Coulombe and Omary, 2002; Fuchs and Weber, 1994). The expression of cytokeratins varies with epithelial cell type, extent of differentiation, and development of the tissue. While amelogenin is the principal postnatal differentiation marker of ameloblasts, the presence of cytokeratin14 (KRT14) in ameloblasts 48–96 h earlier than the expression of amelogenin suggests that KRT14 is a stage-specific differentiation marker for ameloblasts (Tabata et al., 1996). Cytokeratin 14 is a 50 kD polypeptide type I (high molecular weight) keratin that is typically expressed with the type II (low molecular weight) keratin cytokeratin 5.

Ravindranath et al. found an interaction between amelogenin and KRT14 in forming enamel (Ravindranath et al., 2001). KRT14 was found to bind specifically to amelogenins through the conserved tri-tyrosyl motif of amelogenin (ATMP motif). Furthermore, they showed that putative loss of function mutations of ATMP (e.g. substitution of a proline residue with threonine, as noted above) reduces binding of amelogenins to KRT14. Their observations suggested that the ATMP motif at the N-terminal region of amelogenins functions as a signal peptide

for the GMp-ligand of KRT14 and KRT14 may perform a chaperon role during amelogenesis. Confocal microscopy revealed co-assembly of KRT and amelogenin in the perinuclear region of ameloblasts on day 0, with migration of the co-assembled KRT14/amelogenin pair to the apical region of the ameloblasts from day 1, reaching a peak on days 3–5 postnatal, and a collapse of the co-assembly.

As few markers of ameloblast differentiation are known we examined the effect of LRAP overexpression on Cytokeratin 14 protein expression. Immunostaining was performed using a KRT14 monoclonal Ab (D19-N, Abnova Inc, Taipei, Taiwan) in conjunction with the Vector Mouse on Mouse (M.O.M.™) kit (Vector Labs Inc, Burlingame, CA). After application of the secondary antibody supplied in the assay was the same as that described for immunostaining in chapter-3. In both P0 & P5 TgLRAP molar epithelial tissue KRT14 was increased compared to WT (Figure 1). The increased expression at P5 may be the result of a disorganization of cytoskeletal structure and polarization in the TgLRAP model. In the P0 tissue, however, this would be less likely to be an explanation as initial polarization has just commenced.

The reason for the dramatically increased amount of cytokeratin 14 in the TgLRAP mouse ameloblasts is not clear. In our published paper, showing the TgRLAP phenotype, cell polarity, we found that TgLRAP mice had an earlier loss of SATB1 as compared wild type mice. We linked to this earlier loss of SATB1 to

enhanced ameloblast differentiation in the TgLRAP mice as amelogenin proteins were expressed earlier and in greater amounts in the TgLRAP mice. However increased cytokeratin 14 expression suggests ameloblast de-differentiation, suggesting the possibility that LRAP alters multiple transcription pathways in ameloblasts.

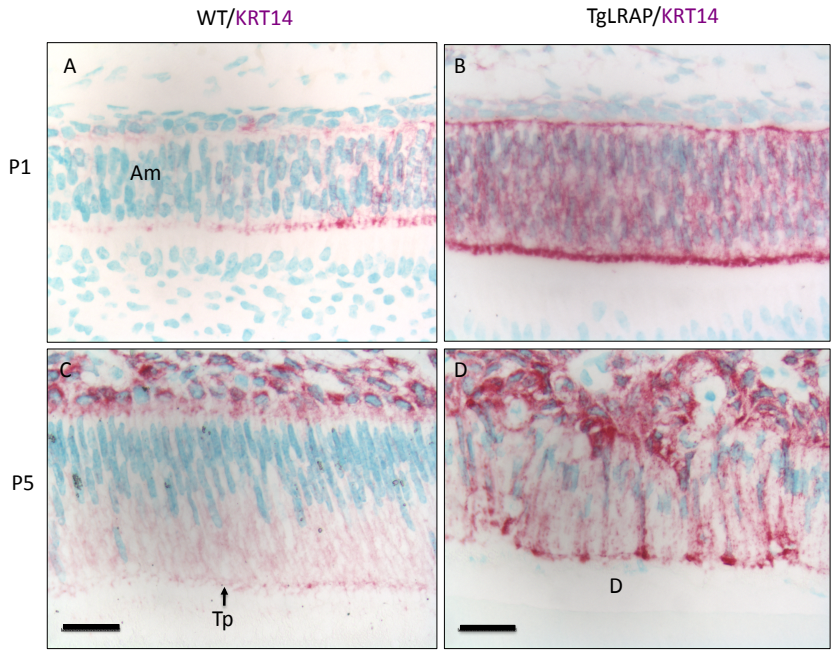


Figure 1. Cytokeratin 14 expression increased in the transgenic LRAP molar epithelium. A) At P0 KRT14 expression in WT molar epithelial tissue was strongest at the apical end of the ameloblasts with decreased expression levels detected in the cytoplasm. B) In comparison TgLRAP pre-ameloblasts showed strong expression in all areas of the cell. C) At P5 expression of KRT14 was primarily localized to the stratum intermedium and stellate reticulum in the WT molar. D) In the TgLRAP molar at P5 expression of KRT14 remained strong in the stratum intermedium and stellate reticulum with strong expression detected at the apical end of the ameloblasts and the lateral surface of the secretory ameloblasts. Scale bar 50 µm for A,B,C,D

LAMP1

Lysosome-associated membrane protein-1 (LAMP-1) binds to LRAP and has therefore been suggested to be a cell surface receptor for LRAP in both epithelial and mesenchymal cells. LAMP-1 is a type 1 integral membrane protein localized primarily on the periphery of the lysosome as a major constituent of the lysosomal membrane (Chen et al., 1986; Chen et al., 1985). Although the majority of LAMP molecules reside in the lysosome, some (<5%) of LAMP-1 and LAMP-2 are also expressed on the cell surface of a variety of cultured cells (Mane et al., 1989). LAMP-1 was initially identified as a transmembrane protein having a structural role in endosomal and lysosomal membranes (Eskelinen et al., 2003). LAMP-1 has also been detected on the plasma membrane.

Tompkins et al. demonstrated that LRAP (M59, A-4) regulates mesenchymal cells (mouse myoblast cell line C2C12) at least partly through LAMP-1. Interactions between amelogenins and LAMP-1 were demonstrated in separate reports using confocal microscopy in which amelogenin isoforms rp(H)M180 or M59 were colocalized with LAMP-1 at the cell surface of LS8 ameloblast-like cells (Iacob and Veis, 2008; Shapiro et al., 2007). However, in a more recent study using a dental epithelial cell line (HAT-7), Xu et al. demonstrated that LAMP-1 is not a receptor for full length amelogenin (M180) recombinant protein on, while LAMP-3 is involved in amelogenin mRNA degradation.

It is important to understand the structure of amelogenin when considering the complex nature of the interaction between amelogenin isoforms and LAMP-1(Margolis et al., 2006). Efforts to determine the exact structure of the amelogenin protein via crystallographic methods have proven unsuccessful. However, investigation into the native structure of amelogenin has shown that the high content of the amino acid proline contributes to its unstructured nature, and has led to the suggestion that amelogenin belongs to a class of proteins known as intrinsically disordered proteins (IDPs) (Delak et al., 2009; Lakshminarayanan et al., 2007; Lakshminarayanan et al., 2009; Tompa, 2002), which do not have a folded structure under physiological conditions. Another feature of IDPs is they can have multiple binding partners or functions and can be involved in signaling pathways (Wright and Dyson, 2009). It has been proposed that the lack of innate global secondary structure of amelogenin may account for its observed roles in both enamel biomineralization and cell signaling (Zhang et al., 2010). IDPs often fold upon binding to their targets and this binding can develop in a stepwise fashion referred to as fly-casting based on their flexible nature (Tompa, 2002; Wright and Dyson, 1999, 2009).

Therefore, it is possible that LAMP-1 functions by binding and maintaining amelogenin in a more structured form in which amelogenin could then bind correctly to a receptor, yet to be identified, which initiates an intracellular signaling cascade which has also yet to be identified. A study by Huang and colleagues used anti-LAMP1 antibody to block the binding of rh174 amelogenin

to LAMP1, while the MAPK inhibitor, U0126, was used to block ERK activity. It was shown that rh174 increased the proliferation of MSCs derived from human bone marrow and MAPK-ERK activity. The MSC proliferation and MAPK-ERK activity enhanced by rh174 were reduced by the addition of anti-LAMP1 antibody suggesting LAMP1 interaction with amelogenin may in part be modulated by the MAPK signaling pathway (Huang et al., 2010).

Immunofluorescent staining of LAMP1 in human fetal enamel organ previously in our lab showed a strong positive staining for LAMP-1 in the stratum intermedium and stellate reticulum of a frozen section of a human enamel organ, while immunostaining for LAMP-1 in ameloblasts was not detectable (Le et al., 2007). The fetal tissue was estimated to be in the bell stage of development. Further work using isolated primary human fetal tooth ameloblasts treated with recombinant human LRAP protein led to an upregulation of LAMP-1 based on immunodetection suggesting it may play an important role in ameloblast differentiation.

I completed immunolocalization of LAMP1 in TgLRAP and WT mouse molars using an antibody obtained from the University of Iowa Hybridoma Bank (1D4B) and was raised in a rat. Immunohistochemistry was carried as previously described with the exception of using a rat secondary antibody (Dako). Polarized and secretory ameloblasts of the WT molar expressed high levels of LAMP1.

Other cells of the developing dental organ which expressed LAMP1 included odontoblasts and stellate reticulum.

In the TgLRAP molar expression level of LAMP1 was overall greatly reduced, at the same time that amelogenin contained within the cells was increased (Figure 2). The strongest expression was detected in the earliest secretory ameloblasts and pre-secretory ameloblasts. This result suggest that as expression of amelogenins is endogenously increased in the cells the function of LAMP1 is decreased along with a concurrent decrease in the cells need to produce the protein. Using quantitative PCR based on whole tooth buds we did not detect a coordinated change in LAMP1 mRNA expression at either P0 or P5.

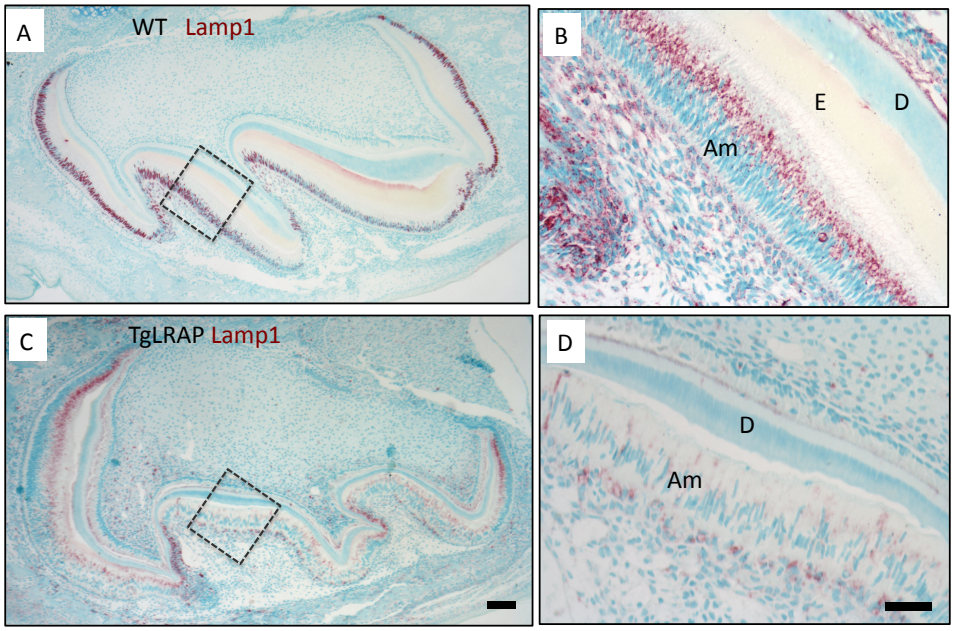


Figure 2: LAMP1 expression was decreased in secretory stage of TgLRAP ameloblasts. A&B) In the WT ameloblasts at P5 LAMP1 expression was expressed at relatively consistent levels within the primarily secretory ameloblasts. Expression in the ameloblasts was well localized in the cytoplasm. C&D). The expression of lamp1 was decreased in all ameloblasts in the TgLRAP molar compared to the early secretory ameloblasts. In the late secretory ameloblasts along the mesial cusp slope (B) expression was strongest at the stratum intermedium. Scale bar 100 μm for A,C; 20 μm for B,D

This study was the first to report the *in vivo* effects of LRAP on the expression of LAMP1. My results, which clearly show LAMP1 expression in ameloblasts differs from the previous *in vitro* work done in our lab by Le et al (Le et al., 2007). However, the difference between the studies is that *in vitro*, exogenous extracellular LRAP was added to the cell culture medium, where as *in vivo* in the TgLRAP overexpressor mice, amelogenins were retained intercellularly. This

suggests that when intracellular amelogenins reach high enough intracellular levels, LRAP is downregulated to limit the update of additional amelogenins intracellularly.

Integrin alpha2 beta1

Integrins are a large family of heterodimeric trans-membrane receptors, an integrin molecule is composed of two noncovalently associated transmembrane glycoprotein subunits called α and β (Hynes, 2002). Integrins serve as the main receptor proteins used by cells to both bind to and respond to the extracellular matrix. Integrins signal through the cell membrane in a bi-directional manner and regulate cellular processes such as cell adhesion, proliferation, migration, and basement membrane assembly (Berrier and Yamada, 2007; Giancotti and Ruoslahti, 1999). Because the same integrin molecule in different cell types can have different ligand-binding specificities, it seems that additional cell-type-specific factors can interact with integrins to modulate their binding activity.

Integrins mediate information from the extracellular matrix (ECM) into the cell in a two-way process that regulates gene expression, cell proliferation, and cell migration. 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. Ameloblasts express α2β1 integrin when they assume their columnar shape (Wu and Santoro, 1994), but no tooth phenotype was observed in α2 integrin

knockout mice and this was likely due to compensation and redundancy of other integrins. 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 it is not evolutionarily conserved (Hu et al., 1997a). Ameloblastin contains binding sites for the RGD motif-binding integrins (Cerny et al., 1996), and it has been shown to bind to ameloblasts, but the receptor has not yet been identified (Fukumoto et al., 2004). $\alpha 2\beta 1$ tends to be expressed around the lateral and apical surfaces of epithelial cells, but it lacks the predominant basal surface expression. $\alpha 2\beta 1$ can interact with collagen IV, the most abundant protein in basement membranes, but it prefers other collagen isoforms, particularly collagen I (Kern and Marcantonio, 1998).

Immunostaining showed that in the TgLRAP molar at the secretory stage, $\alpha 2\beta 1$ protein was increased in the ameloblasts (Figure 3). These results were similar to the upregulation of cytokeratin 14 and amelogenin contained within the TgLRAP ameloblasts. The TgLRAP mice demonstrate a marked increase in protein detected in all areas of the ameloblast including the basal surface. WT teeth appeared to have staining only in the apical regions of the cells. Interestingly our lab (Yan Zhang, unpublished work) also showed that the SATB1 knock out mice which have a similar alteration of cytoskeletal structure compared to WT as does the TgLRAP model mice also showed a marked increase in this integrin. This

may suggest that an LRAP mediated decrease in SATB1 leads to increased integrin synthesis, which results in the alterations in ameloblast differentiation discussed in chapter X. The dramatic increase of $\alpha 2\beta 1$ integrin throughout the ameloblasts in the TgLRAP mouse molars is likely to contribute to the loss of polarity by these cells.

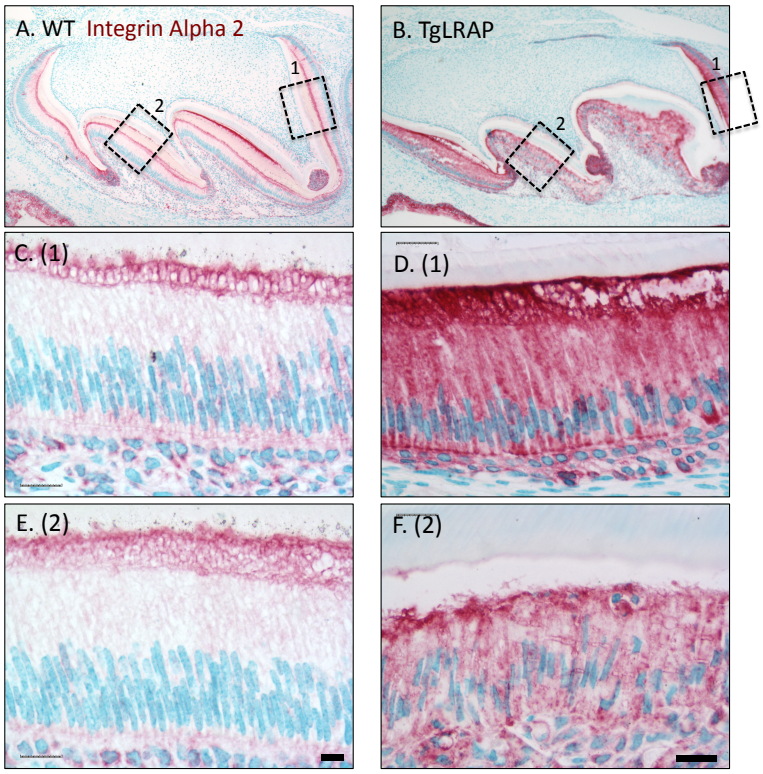


Figure 3. Expression of integrin α2β1 protein is increased in early and late secretory ameloblasts. A,C, & E) Expression of α2β1 is strongest in the WT P5 molar at the apical end of the ameloblasts in the are of the Tomes' processes. Expression is also detected in the stratum intermedium. B, D, & F) Expression of α2β1 in the TgLRAP P5 early and late stage ameloblasts is stronger in all areas compared to WT tissues. The early stage ameloblasts do not demonstrate the loss of cytoskeletal organization that is seem in the later stage cell over the cuspal slopes. Scale bar 100 μm for A,B; 20 μm for C,D,E,F

MMP20

Post-secretory processing of amelogenins involves a series of discrete steps including supramolecular self-assembly and progressive proteolytic reduction in molecular size, facilitating enamel biomineralization and maturation (Fincham et

al., 1999). During enamel formation almost all of the protein supporting mineral formation is removed. It has been previously demonstrated that two proteases are involved in matrix hydrolysis: matrix metalloproteinase 20 (Bartlett et al., 1996) and kallikrein 4 (Simmer et al., 1998). MMP-20 is a protease that is secreted within enamel proteins during the early (secretory) stage of amelogenesis, when the crystallites are growing predominantly in length. MMP-20 cleaves the enamel proteins and selected enamel protein cleavage products are reabsorbed by ameloblasts and degraded (Lu et al., 2008).

In chapter 3 we demonstrated that at P0 (pre-ameloblasts) there was a large 200 fold in MMP20 mRNA expression. An antibody specific to MMP20 was employed on the TgLRAP histological sections to determine the protein expression and spatial localization of MMP20. Immunoreactivity was assayed as previously described using a rabbit polyclonal anti-matrix metalloproteinase-20 antibody (M5934, Sigma Aldrich Inc, St Louis, MO.). Although the antibody was specific to MMP20 it highly likely that it detects other MMPs as well. Ameloblasts in the WT mouse showed typical organization of enamel matrix ameloblast layer. I completed immunohistochemical localization of MMP20 (as well as other MMPs, and found what appeared as amorphous aggregates of MMP protein appeared in cyst-like structures between ameloblasts (Figure 4 B&D). The accumulation of MMPs is parallel to the increased accumulation of amelogenin both between the cells and within the cells.

I subsequently stained undemineralized sections of mouse molars with von Kossa reagents to detect mineralization followed by counterstaining with Toluidine Blue (see chapter-4 for methodology). Compared with WT molars, TgLRAP mice show accumulation of mineralized extracellullar matrix between the ameloblast cells that appear to coincide with areas of increased MMP20 secretion (Figure 4 A&C). This suggests MMP20 protein is specifically synthesized by cells that synthesize amelogenin, and interactions between MMP20 and amelogenin result in the formation of mineral.

Although our data agrees with that of others that LRAP appears to have little effect on crystal growth within the forming enamel matrix LRAP may have a critical role in indirectly modulating amelogenin and MMP-20 synthesis, leading to mineralizatin of the enamel matrix. Additional studies to determine the up-stream pathways through which LRAP controls the expression of matrix related genes warrant further investigation.

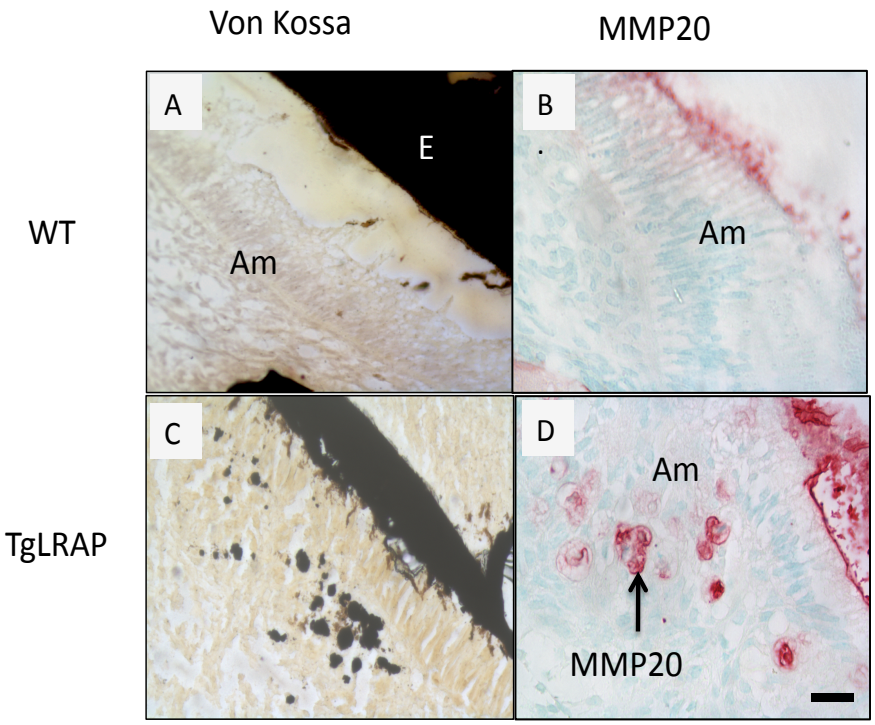


Figure 4. (A&B) von Kossa staining and MMP20 expression of secretory stage WT mouse molar shows a mineralized enamel matrix with MMPs only detected at the apical region of the ameloblasts. (C&D) Accumulation of amorphous matrix material between the ameloblast layer and the forming enamel, and between ameloblasts, in TgLRAP mice with MMP accumulations detected in multiple areas within the ameloblast layer as well as strong expression in the matrix. Scale bar 30

WNT Expression

Multiple Wnt family members as well as a large number of effectors in their pathway have important roles during the development of the enamel organ (Thesleff and Mikkola, 2002; Tucker and Sharpe, 2004). The canonical Wnt

pathway is activated when β-catenin enters the nucleus and interacts with TCF/LEF transcription factors to promote downstream gene expression.

Multiple studies have suggested that LRAP may affect the Wnt signaling pathway, and in a recent report Wen and co-workers suggested that LRAP may promote osteogenesis through the Wnt pathway. Specifically, LRAP treatment elevated Wnt10b, a canonical Wnt/β-catenin signaling molecule, expression level whereas Wnt10b knockdown by siRNA abrogates the effect of LRAP (Wen et al., 2011). Another study reported that LRAP activates the canonical Wnt signaling pathway to induce osteogenic differentiation of mouse ES cells (Warotayanont et al., 2009). Developmentally Wnt10b was expressed specifically in cells of the putative molar dental epithelium starting at the bud stage (Sarkar and Sharpe, 1999).

Therefore, we examined what effect LRAP would have on the expression of Wnt10b in the developing postnatal enamel organ. We found that similar to osteoblasts that LRAP overexpression led to an increase in Wnt10b expression in the late secretory stage ameloblasts of the TgLRAP molar compared to the WT molar (Figure 5). Expression of Wnt10b in the odontoblasts could not be detected. Our finding suggests that it is possible the mechanism through which LRAP regulates differentiation of ameloblasts is Wnt signaling in addition to other potential pathways.

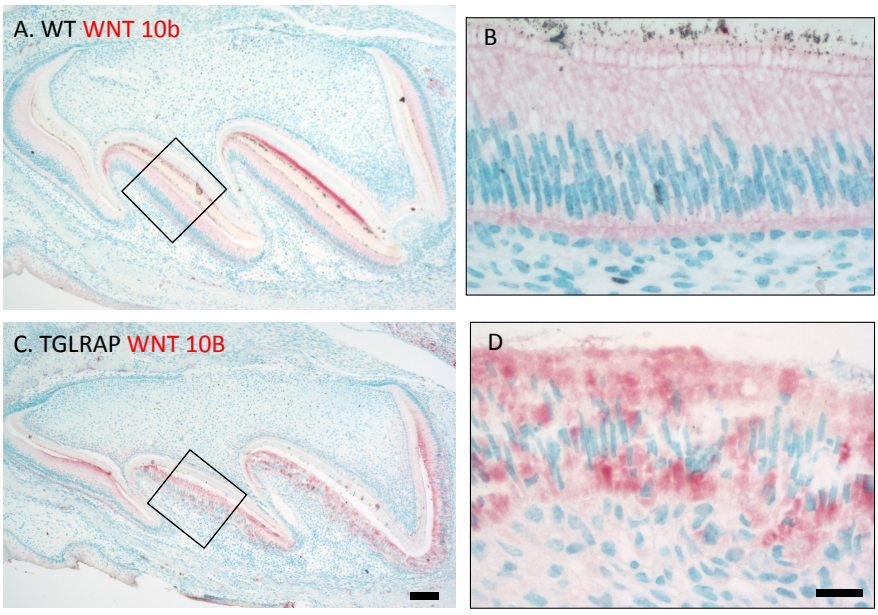


Figure 5. LRAP overexpression increased WNT 10b protein expression in molar ameloblasts. A&B) Expression of the canonical Wnt10b signaling ligand was minimally detected in the ameloblasts of the P5 WT molar with no expression detected in the odontoblasts. C&D) In the TgLRAP P5 molar expression was more intense in the ameloblasts with no expression detected in the odontoblasts. Scale bar 100 μm for A,C; 20 μm for B,D

TgLRAP/M180 Phenotype

Previously Gibson et al (Gibson et al., 2011) determined the results of LRAP and M180 overexpression together in the amelogenin null mouse model. The result was that the co-expression led to a small improvement in the rescue of the amelogenin matrix in comparison to M180 alone.

To see if the ameloblast and matrix phenotype demonstrated in the TgLRAP mouse in the WT background would be affected by co-expressing both TgLRAP and TgM180 a cross mating of the two transgenic mice with each other was performed and pups were collected at postnatal days 5 and 10 (Figure 6). At P5 normal enamel matrix was observed, but an ameloblast phenotype was still present in the late secretory ameloblasts as previously observed. The expected outcome was that a more severe phenotype would exist than was detected in the TgLRAP mouse alone (Chapter 3), however that did not occur. This outcome was expected because the uncontrolled overexpression of amelogenins (multiple isoforms) would occur in a model in which full length amelogenin was already being overexpressed. I hypothesize that the more subtle phenotype noted is the result of a possible feedback loop where the overexpression of M180 from the M180 transgenic mouse controlled, to an extent, the seemingly uncontrolled overexpression of M180 previously detected.

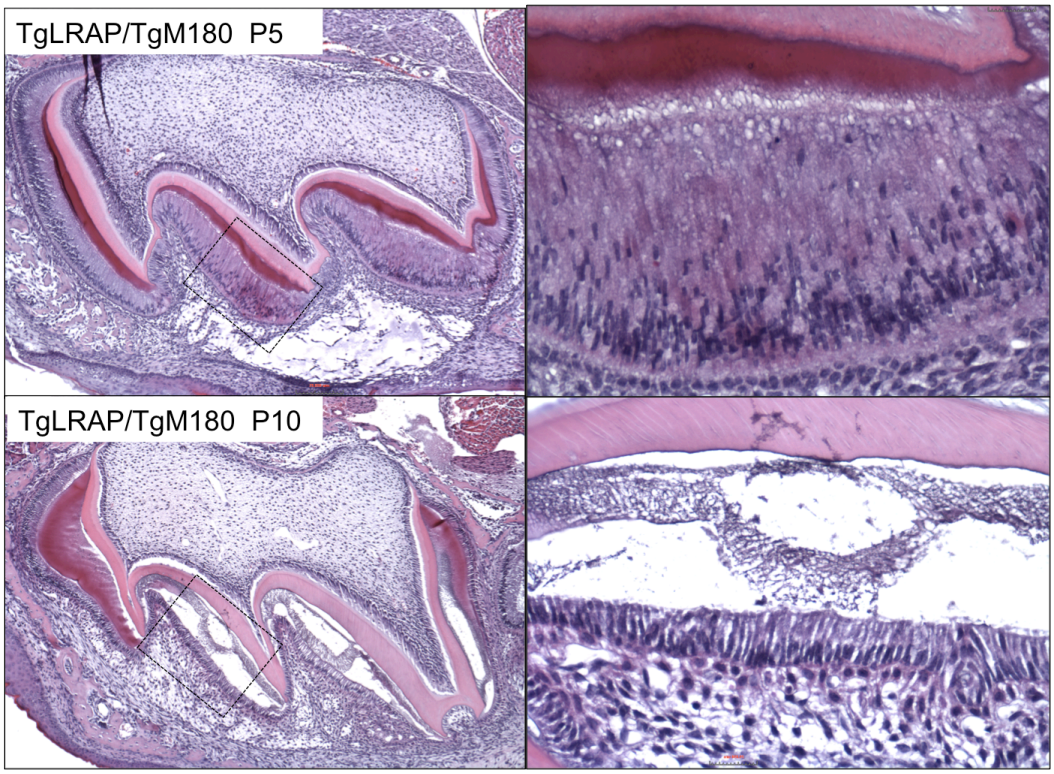


Figure 6: Simultaneous overexpression of LRAP and M180. A&B) At P5 a phenotype characterized by disorganization of the ameloblast over the mesial cusp slope. An enamel matrix was laid down both on the cuspal and approximal surface of the tooth. C&D) At P10 the columnar ameloblasts shortened into maturation stage ameloblasts which presented with a disorganized appearance.

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Chapter 4. Exon4 Transcribed Amelogenins Influence Enamel Biomineralization

Abstract

Alternatively spliced amelogenins derived from transcripts containing exon4, are considered to have low abundance with an unknown function in enamel matrix biomineralization. In this study we determined that amelogenin mRNAs with exon4, and amelogenin proteins with the translated exon4 amino acid sequence, are upregulated at the late secretory stage of amelogenesis. In mice overexpressing LRAP, where ameloblast differentiation and mineralization occurs earlier, we also found an earlier upregulation of amelogenins with exon4. Bioinformatics analyses predicted that exon4 translated peptide has enhanced binding to apatite, and given the location of amelogenins with exon4, it may be that amelogenins with exon4 limit mineral apposition of hydroxyapatite crystals at the early maturation stage while protein is rapidly removed prior to final mineralization. We further determined that the exon4 sequence is a miRNA with reduced expression at the late secretory stage when amelogenins containing exon4 translated sequences were upregulated. These results suggest multiple functions for exon4 in mediating both cell differentiation and matrix mineralization during enamel formation.

Introduction

Amelogenins constitute the major protein component of the secretory dental enamel matrix (Termine et al., 1980). Amelogenins are required for enamel matrix formation and biomineralization, as mouse models lacking the amelogenin transcript do not form a functional mineralized enamel matrix (Gibson et al., 2001)

Exon4 is present in the amelogenin gene of many species (Sire et al., 2012), and has been identified in human (Salido et al., 1992), mouse (Simmer et al. 1994) and rat (Li et al, 1995) mRNAs. Differential expression of amelogenins containing exon4 is indicated by studies reporting expression of M73 (leucine rich amelogenin peptide plus exon4) in odontoblasts, and M194 (the most dominant near full length amelogenin, M180 plus exon4) in stratum intermedium, but not in ameloblasts (Iacob and Veis, 2006). Amelogenin proteins containing exon4 transcripts have been reported in mouse (Simmer 1994) and porcine (Yamakoshi, 2011) enamel matrix.

A number of studies have shown different signaling properties of M59 (leucine rich amelogenin peptide, LRAP) and M73 in pulp repair (Goldberg et al., 2003) and chondrogenesis (Tompkins and Veis, 2002), suggesting the possibility that alternatively spliced amelogenins containing exon4 transcripts may also have a unique function in enamel formation. The purpose of this study was to determine the potential role of alternatively spliced amelogenins containing exon4 in enamel formation. Our previous work (Stahl et al., 2013a) showed that in mice overexpressing LRAP, ameloblasts

differentiated earlier, and we further used this model to investigate the relationship between exon4 transcribed peptides and ameloblast differentiation.

Methods

Mass spectrometry (MS) analysis of amelogenins in the developing rat enamel matrix

All animal procedures were done with approval by the University of California San Francisco and University of Pennsylvania Institutional Animal Care and Use Committees. Nine week old Wistar female rats were sacrificed by CO_2 inhalation followed by cervical dislocation. Mandibular incisors were removed from the alveolar bone, and enamel matrix was dissected from the underlying dentin surface as previously described (Den Besten, 1986; Smith and Nanci, 1989) (Fig 1A).

Enamel proteins were extracted in 0.5 M acetic acid with protease inhibitor cocktail (Roche, Indianapolis, IN), and then centrifuged at 10,000g for 10 minutes to remove the undissolved particles. The protein was desalted on a C18 Zip Tip (Millipore, Billerica, MA), and mixed at 1:1 volume ratio with α-Cyano-4-hydroxycinnamic acid in 50% MeCN/0.1%TFA matrix. Mass spectrometry was done on a Voyager DE STR MALDI-TOF MS (Applied Biosystems, Foster City, CA).

Comparison of predicted calcium and phosphate binding sites in amelogenin isoforms

We predicted calcium and phosphate binding sites in amelogenin isoforms with and without exon4 by calculating function-specific meta-functional signatures (Horst and Samudrala, 2010; Martinez-Avila et al., 2012). These meta-functional signatures represent the output of protein sequence analytic machine learning algorithms that derive

a likelihood of calcium and phosphate binding activity for each residue in a protein based on proteins with known binding activities (Horst et al., 2010). One-dimensional predictions of conservation and structural features were combined, by training logistic regressions to maximize scores for 591 amino acid residue side chains noncovalently bound to phosphate ions, among 39,641 residues in crystal diffraction structures of 190 nonredundant proteins in the Protein Databank (Berman et al., 2000; Horst and Samudrala, 2010; Martinez-Avila et al., 2012) , and 696 calcium binding residues among 72,355 in 294 protein structures (Horst and Samudrala, 2010). Each method was extensively tested by cross-validation and application to non-redundant sets. A threshold of 3 standard deviations was found to remove nearly all false positives upon application to 1000 proteins without significant sequence similarity to phosphate- or calcium-binding proteins, respectively.

Mouse models

TgLRAP mice were made by incorporating a plasmid containing the amelogenin promoter with cDNA encoding for LRAP (TgLRAP) (Chen et al., 2003). Wild type (WT) mice were generated as littermates and sacrificed at same time point as controls.

Histomorphology and immunohistochemistry

Maxillae were dissected from mice sacrificed by CO_2 inhalation followed by decapitation, and immersed into 4% paraformaldehyde (PFA) for 1 day at 4 °C, followed by decalcification in 8% EDTA (pH 7.3) at 4 °C for 10 days. The samples were then dehydrated through a graded series of ethanol followed by routine paraffin embedding

and sectioning. After deparaffinization, sections were stained with Gomori's Trichrome or immunostained as previously described (Stahl et al., 2013a) using a rabbit anti-exon4 antibody. The rabbit anti-exon4 polyclonal antibody was raised against murine amelogenin exon4 peptide (Genscript Inc. Piscataway NJ), and IgA was purified by Protein A affinity chromtography. Negative controls were incubated with preimmune IgA.

Von Kossa staining

Maxillae from P5 WT and TgLRAP mice were fixed as described above, and undecalcified frozen sections were obtained as previously described (Nakano et al., 2004). Mineralization of the tissue was visualized by 2.5% silver nitrate staining (von Kossa staining) (Bleicher et al., 1999).

PCR amplifaction of amelogenin mRNA with or without exon4.

Total mRNA was isolated from P0 and P5 WT and TgLRAP first maxillary molars using RNeasy Mini kit (Qiagen, Germantown, MD). An aliquot containing 1µg of total RNA was reverse transcribed using SuperScript® III Reverse Transcriptase (Invitrogen, Carlsbad, CA) followed by PCR reaction using Hot Start Taq kit (Qiagen) (see Appendix Table 1 for primers and condition of PCR).

MicroRNA analyses

Total RNA including miRNA was purified from P0 WT and TgLRAP maxillary first molars using the Qiagen miRNeasy kit (Qiagen) followed by fraction isolation for

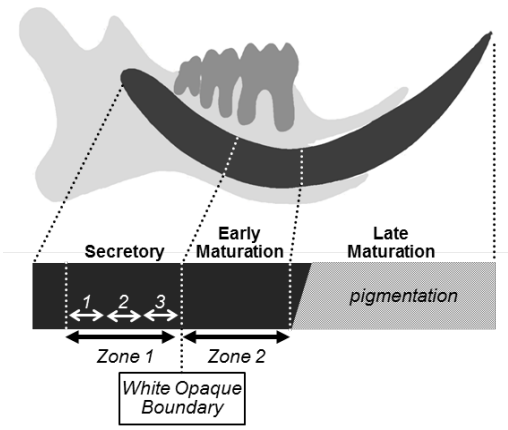
enrichment of miRNAs and other small RNAs (less than ~200 nt) using RNeasy MinElute Cleanup Kit (Qiagen). For selective conversion of mature miRNAs into cDNA, reverse transcription was performed using miSript II RT kit (Qiagen). A custom primer for putative murine amelogenin exon4 miRNA sequence (5' acugacaggacugcauu 3'), and primers for mmu-miR-23a and -23b and has-miR-3660 was obtained from Qiagen. MicroRNA expression was characterized by quantitative PCR with the miScript SYBR Green PCR Kit (Qiagen) using the ABI 7500 system (Applied Biosystems). RNU6B was used as a reference target gene. Relative expression levels of target genes were analyzed by the ΔΔCt method as published previously (Thomsen et al., 2010). All data were analyzed by *student* t-test by using Prism software (GraphPad Software Inc, San Diego, CA, USA).

Results

Amelogenins coded by exon4 were identified only in transition/early maturation stage rat enamel matrix

Amelogenin fragments isolated from normal rat secretory (zone 1), and early maturation (zone 2) had the same cleavage sites. The exon4 peptide sequence was only present in amelogenin fragments isolated from zone 2 (Fig 1).

A



B

MGTWILFACLLGAAFAMPLPPHPGS
PGYINLSYE**KSHSQAINTDRTALVLT**
PLKWYQSMIRQPYPSYGYEPMGG
WLHHQIIPVLSQQHPPSHTLQPHH
HLPVVPAQQPVAPQQPMMPVPGH
HSMTPTQHHQPNIPPSAQQPFQQP
FQPQAIPPQSHQPMQPQSPLHPM
QPLAPQPPLPPLFSMQPLSPILPELPL
EAWPATDKTKREEVD

C

Fragment (- Exon 4)	Zone	Fragment (+ Exon 4)	Zone	Cleavage Site
MPLPPHPGSPGYINLSY EVLTPLKWYQS	1-3	MPLPPHPGSPGYINLSY E**KSHSQAINTDRTALVLT** PLKWYQS	2	S/M
MPLPPHPGSPGYINLSY EVLTPLKWY	1-3	MPLPPHPGSPGYINLSY E**KSHSQAINTDRTALVLT** PLKWY	2	Y/Q
MPLPPHPGSPGYINLSY EVLTPL	1-3	MPLPPHPGSPGYINLSY E**KSHSQAINTDRTALVLT** PL	2	L/K
MPLPPHPGSPGYINLSY EVLTP	1-2	MPLPPHPGSPGYINLSY E**KSHSQAINTDRTALVLT** P	2	P/L

Figure 1: Expression of amelogenin fragments containing exon 4 in enamel matrix. (A) Secretory (Zone 1) and early maturation (Zone2) enamel matrix was dissected as denoted on the incisor image. Zone1 was further divided into three areas (Zones 1-1, 1+2 and 1-3). Zone2 includes the maturation stage with both zones overlapping into the transition stage. (B) The protein sequence for the full-length amelogenin isoform containing 194 amino acids with the sequence derived from exon4 highlighted in red. (C) The list of the protein fragments with and without exon4 detected by MS. In all cases, the fragments containing exon 4 were expressed in Zone2. The protein fragments of matching sequence but lacking exon4 were detected in Zone1-2 or 1-3.

Both protein and mRNA of amelogenins with exon4 were detected earlier in TgLRAP mouse teeth as compared to WT

In P5 WT molars, exon4 positive immunostaining was visible only at the apical border of the late secretory ameloblasts (Fig 2A, a-c). In TgLRAP mice, amelogenins with exon4 translation were synthesized earlier in ameloblasts, which showed intense intracellular amelogenin immunoreaction at the late secretory stage (Fig 2A, d-f).

At P0 mRNA transcripts containing exon4 (M194) were amplified in TgLRAP, but not WT molars (Fig 2H lanes 1&2) mice. At P5 both WT and TgLRAP expressed M194 (Fig 2H lanes 3&4).

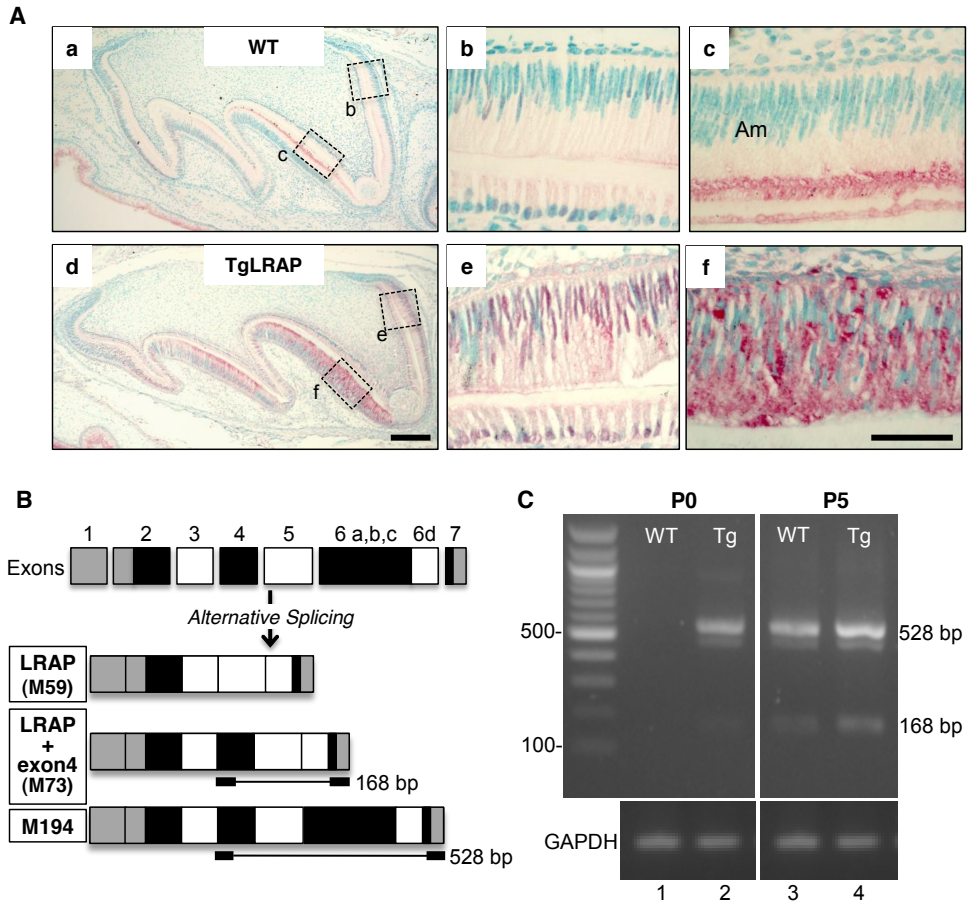


Figure 2: Expression of amelogenin variants containing exon4. (A) Immunolocalization of amelogenin with exon4 in P5 WT and TgLRAP molar. In the WT molar (a-c), amelogenin with exon4 was present at the apical border of the secretory ameloblasts, with staining primarily localized to the late secretory stage cells (c) but not at pre-secretory ameloblasts (b). In contrast to WT, TgLRAP molar (d-f) showed expression of amelogenin with exon 4 beginning earlier in the pre-secretory stage (e) and expressing more intensively in the entire cytoplasm of secretory stage ameloblasts (f). (B) The alternatively spliced isoform LRAP (M59) lacks exon 4 and the exon 6 a,b,c domain. M73 is an isoform consists of LRAP and exon4. M194 is a full length isoform. A PCR primer set starting exon4 and exon7 was designed to detect amelogenin variants containing exon4. (C) Expression of amelogenin mRNA variants with exon4 in WT and TgLRAP molar. At P0, isoforms with exon4 were only identified in the TgLRAP mouse (lanes 1&2). At P5, the two major bands (528 bp and 168 bp) were observed in both WT and TgLRAP mice (lanes 3&4). Scale bar 100 μm for A,D;

Overexpression of LRAP and earlier synthesis of amelogenins with exon4 corresponded to earlier ameloblast differentiation and matrix mineralization.

Von Kossa staining of P5 molars showed decreased thickness of the mineralized layer in the TgLRAP mice compared to the WT (Fig 3A&E). In Trichrome stained molars (Fig 3B&F), the early secretory stage enamel matrix was stained in red in both WT and TgLRAP indicating the matrix was rich in enamel matrix proteins including amelogenins at this stage (Fig 3C&G). In contrast, enamel matrix in the late secretory stage of the TgLRAP stained blue typical of early maturation stage enamel matrix, but remained red in the WT, indicating that the enamel matrix of TgLRAP molar was in a more advanced stage than WT (Duailibi et al., 2004) (Fig 3D&H). Von Kossa of enamel matrix within the late secretory ameloblast layer (Fig 3F) was intense in TgLRAP mice, where synthesis of amelogenins with exon4 was the highest (Fig 3A-f), suggesting the possibility that amelogenins containing exon4 can enhance enamel biomineralization.

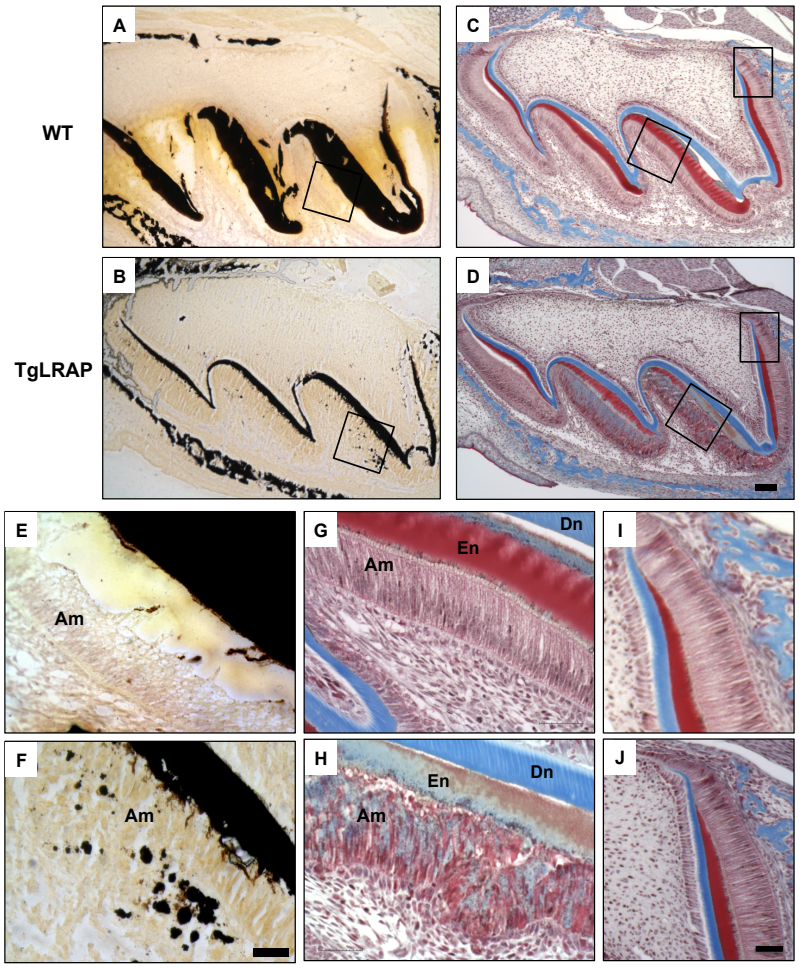


Figure 3: P5 TgLRAP mice exhibit an altered mineralization of enamel matrix at the late secretory stage of ameloblast development. (A, B, E and F); Von Kossa staining showing mineralized deposits as black. (C, D and G-J); Trichrome staining. Von Kossa stained TgLRAP molar indicated a decreased mineralized layer thickness in the entire molar (A&B). In Trichrome stained molar (C&D), the TgLRAP enamel matrix along the distal of both the mesial and central cusps had a decrease in the red staining compared to the WT indicating a loss of enamel matrix layer. In the late secretory ameloblasts along the cuspal slope (E-H), the enamel matrix of WT continue to be red stained by Trichrome (G), whereas the TgLRAP enamel matrix was stained pale blue (H). The late secretory ameloblasts of TgLRAP was disorganized, and the spread of the areas stained similar blue color to the enamel matrix was observed (H). In the late secretory ameloblasts layer of TgLRAP mice also demonstrated a distribution of the Von Kossa positive mineral deposits (F). There was no difference in the staining pattern of the enamel matrix in the earlier secretory stage between WT and TgLRAP (H&J). Scale bar 100 μm for A,B,C,D; 20 μm for E,F; 30 μm for G,I; 20μm for G,H,I,J.

The addition of exon4 to LRAP is predicted to strengthen interactions with calcium and phosphate

The meta-functional signatures analysis predicted functional similarity between specific residues of LRAP and LRAP+4 to calcium and phosphate binding sites in the crystallographic structures of other proteins (Figure 4). The differences in functional signatures for LRAP with (Fig. 4 lower panel) or without the exon4 encoded sequence (Fig. 4 upper panel), suggest exon 4 translated amino acid increase LRAP binding to calcium and phosphate. Within the region encoded by exon4, asparagine 26 and aspartate 28 (in exon4) are predicted with high confidence to bind a single calcium ion, perhaps along with asparagine 14 and glutamate 18. Phosphate binding is predicted throughout residues 35 to 45, only when exon4 is present. Calcium and phosphate interactions predicted for residues 12-32 are consistent with previous experimental data (Gungormous et al., 2012).

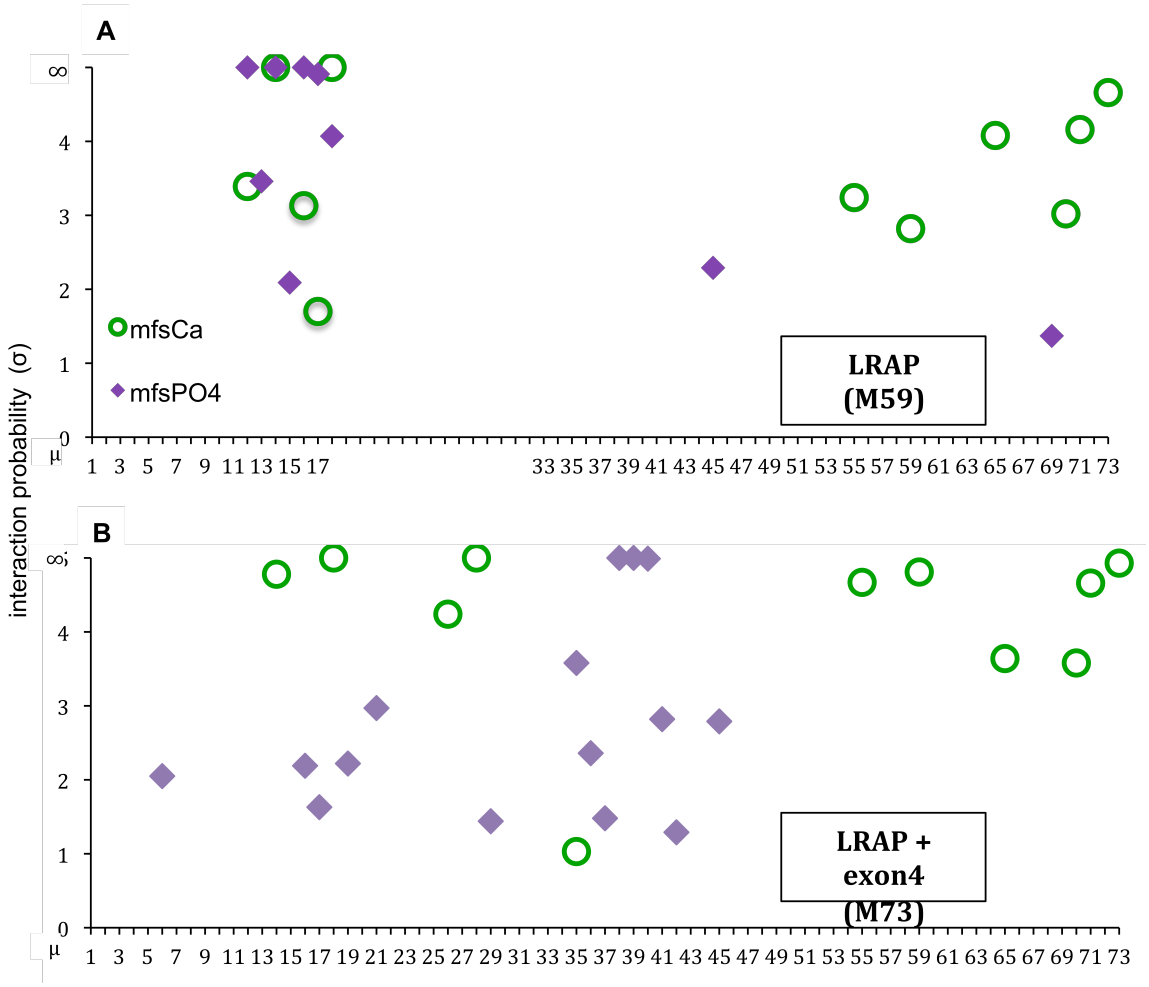
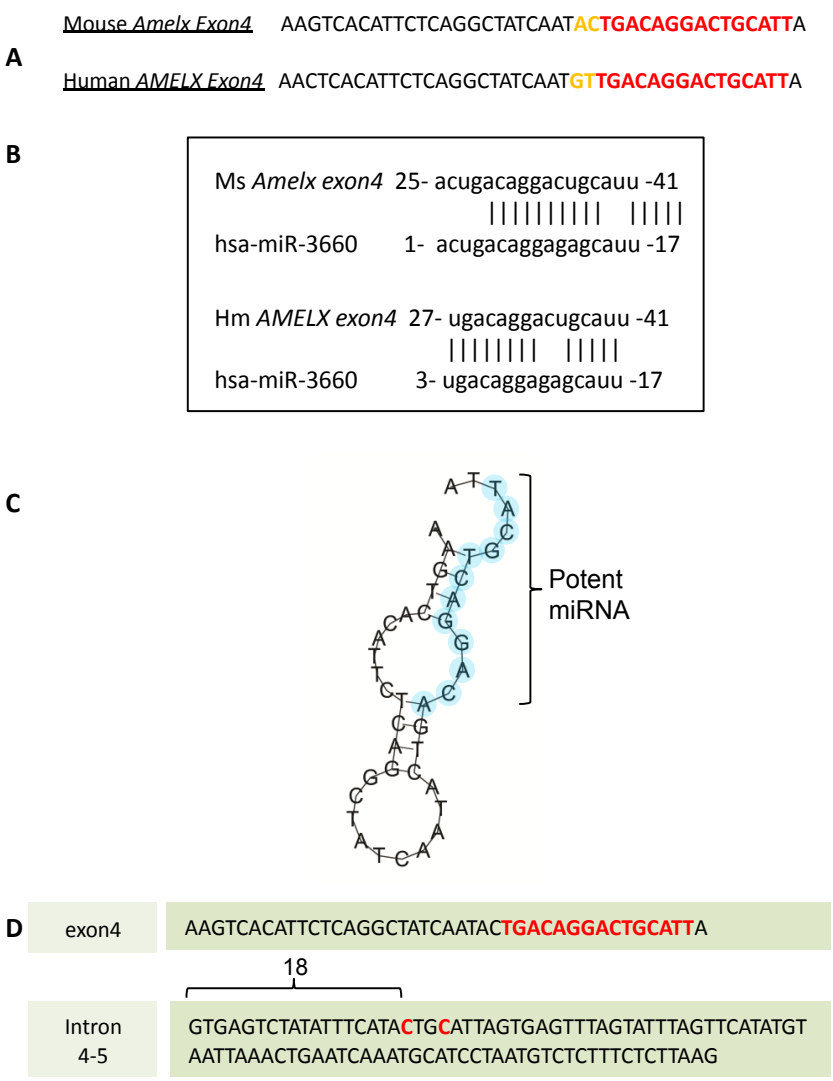


Figure 4: The amelogenin region encoded by exon4 is predicted to interact directly with calcium and phosphate. Calcium- (green) and phosphate-specific (purple) protein meta-functional signatures were calculated for LRAP with (lower) or without (upper) the exon4 encoded sequence. Interaction probability scores are shown for each residue with significant predictions. Predicted structural changes observed with the addition of exon 4 appear to drive interactions with soluble phosphate by residues 34-45 of LRAP+4 and reduce the contribution of residues 12-18 to calcium and phosphate interactions.

Mature miRNA corresponding to exon4 was upregulated in TgLRAP mice

To investigate the possible role of miRNAs in mediating changes in ameloblast differentiation and regulation by isoforms with and without exon4, in the TgLRAP mice, we completed qPCR analysis of putative miRNAs derived from murine amelogenin exon4 as well as mmu-miRNA-23a and -23b, and has-miR-3660 (Supplemental 1). The

two miRNA, mmu-miRNA-23a and -23b, have been identified in developing tooth (http://bite-it.helsinki.fi/) and also are associated with regulation of Special AT-Binding Protein 1 (SATB1) (von Schack et al., 2011), which we found to be downregulated in TgLRAP molars (Stahl et al, 2013. These miRNAs, were not significantly different in WT as compared to TgLRAP P5 mouse molars. The hsa-miR-3660, derived from the noncoding region of chromosome 5 with a high homology to putative amelogenin exon4 miRNA (analyzed at miRBase (Kozomara and Griffiths-Jones, 2011)), was also not significantly different in TgLRAP and WT mice. However the putative miRNA derived from exon4 sequence of amelogenin pre-mRNA, was present in ameloblasts, and was down regulated 4.3 fold in TgLRAP mice as compared to WT mice (p-value < 0.01).



Supplemental Figure 1: Prediction of amelogenin exon4 miRNA (A) cDNA sequence of mouse and human amelogenin exon4. Putative miRNA sequence is in red. (B) Sequence homology between amelogenin exon4 RNA (mouse and human) and hsa-miR-3660. (C) Secondary structure of mouse exon4 RNA predicted at The Vienna RNA Websuite. (D) Presence of CNNC sequence at 18+1 nucleotide downstream of the putative miRNA (in red) in mouse amelogenin cDNA sequence.

Discussion

In these studies, we found that amelogenin mRNA containing exon4 was present in late secretory ameloblasts found in P5 WT mouse molars. Consistent with these results, amelogenin proteins containing exon4 were immunolocalized in the late secretory ameloblasts. We found the peptides containing exon4translated sequence in transition/early maturation enamel matrix, together indicating that amelogenins with exon4 transcript are upregulated at the end of the secretory stage where elongation of enamel crystals is complete.

To gain further insights into the function of amelogenins containing exon4, we compared the pattern of exon4 expression in WT to TgLRAP mice. We previously found that LRAP overepression on a WT background results in earlier amelogenin expression, earlier ameloblast differentiation, and retention of amelogenins in late secretory ameloblasts (Stahl et al., 2013a). In this study, we found that LRAP overexpression also resulted in earlier expression of ameloegenin with exon4 mRNA detected at P0, with corresponding immunopositive staining for amelogenins containing exon4 starting on the pre-secretory and early secretory ameloblasts.

The enamel matrix of the TgLRAP mice mineralized earlier, as shown by Von Kossa staining. These results potentially suggest that amelogenins containing exon4 might enhance mineralization. The calcium and phosphate meta-functional signatures derived for LRAP with or without exon4 (M59 or M73) demonstrated a high likelihood the exon4 part directly interacts with calcium. The one-dimensional predictions of structure and

evolutionary relationships that underlie the meta-functional signature analysis predict a conformational shift to occur throughout residues 10-45 in the presence of exon4, altering the physicochemical environment of residues 33-42 to facilitate interactions with phosphate. Together these data suggest that exon4 works as a module within LRAP to directly alter its relationship to calcium, phosphate, and forming hydroxyapatite.

In vitro, amelogenins have been shown to both promote mineralization and inhibit mineral formation, depending on the environment (Aoba et al., 1987; Beniash et al., 2005; Habelitz et al., 2004). However, *in vivo*, amelogenins modulate crystal growth to allow elongation of enamel crystals, possibly by binding to apatite surfaces to inhibit lateral crystal growth (Wen et al., 1999). The secretion of amelogenins containing exon4 corresponding amino acid sequence at the early maturation stage could potentially spatiotemporally inhibit crystal growth but would allow rapid removal of hydrolyzed peptide fragments to facilitate matrix mineralization at following maturation stage, which is consistent with our previous work that presented a decrease in final mineralization of enamel in the TgLRAP molar (Stahl et al., 2013a). This possibility is further supported by our current observation of early progression of mineralization in TgLRAP enamel matrix even with some ectopic mineral deposits similar to where amelogenin with exon4 protein localized.

We also questioned whether the specific upregulation of amelogenins containing exon4 at the late secretory may have intracellular roles possibly in the initiation of the transition stage of ameloblast formation. We investigated microRNAs as one of the possible

mechanisms that amelogenin exon4 may contribute to intracellular activity. Mature microRNAs (miRNAs), are single-stranded, 18-24 nucleotide non-coding RNAs, formed by from a unique hairpin structure of the pre-mRNAs. The miRNAs can bind to other pre-mRNAs to modulate protein synthesis, resulting in alterations including development, apoptosis, and cell cycle regulation (Bueno et al., 2008; Reinhart et al., 2002; Xu et al., 2003) including tooth development (Jheon et al., 2011; Michon et al., 2010; Sehic et al., 2011). Interestingly amelogenin exon4 has the characteristics of an miRNA including that it : 1) is short (14 amino acids) with possible hairpin secondary structure (simulated by Vienna RNA version2.0) (Lorenz et al., 2011), 2) is spliced out most of the time, 3) has a sequence with 57th percentile score compared to a known miRNA (hsa-miR-3660), and 4) has a splicing factor SRp20-binding motif (CNNC) in vicinity downstream (16-18 nt) (Auyeung et al., 2013), suggesting the possibility that when spliced out, the exon4 sequence may act as a miRNA.

While investigating the possibility of LRAP controlling differentiation of ameloblasts by modulating miRNA expression in ameloblasts, we looked for changes in mmu-miR-23a and -23b as well as hsa-miR-3660, and we did not find alterations in expression of these miRNAs in the TgLRAP mice. However, we did find an upregulation of a putative miRNA derived from the exon4 sequence. Taken together these results suggest that LRAP mediated ameloblast differentiation results in an earlier synthesis of amelogenin splice variants containing exon4 peptide with a concurrent loss of the exon4 derived miRNA, which may mediate ameloblast differentiation.

In conclusion, these studies show that amelogenin splice variants containing exon4 are temporally and spatially upregulated at the late secretory/early transition stage of enamel matrix formation. In mice that over express LRAP, with a resultant earlier ameloblast differentiation, amelogenins containing exon4 are also upregulated, and correlate to early matrix mineralization. Finally, we identified an exon4 derived miRNA, which was downregulated as synthesis of amelogenin mRNA containing exon4 increased, suggesting a possible role of this miRNA in ameloblast differentiation. Future studies will further elucidate the role of amelogenins containing exon4 in enamel matrix biomineralization, and the role of exon4 miRNA in ameloblast differentiation.

Appendix Table1: RT-PCR primers used in amelogenin exon4 analysis

Primer Sequence	Annealing temperature/cycles
F: 5'-AAGTCACATTCTCAGGCTATCAATACT-3' R: 5'-GTCCACTTCTTCCCGCTTG-3'	57 °C/30

Reference:

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Chapter 5. Incisor microCT

Mouse incisors differ from molars in that they grow continuously throughout life with enamel deposition uniquely on the outer, or labial, side of the tooth as ameloblasts differentiation is restricted to that area (Warshawsky, 1968). Some scientists have suggested that stem cells which are responsible for the incisors continuous growth are found in an area termed the cervical loop at the posterior end of the incisor which encompasses both epithelial and mesenchymal cells (Gronthos et al., 2002; Harada et al., 1999). I initiated this project started with a micro CT analysis incisors from TgLRAP/WT and TgLRAP/Amelx Null models in comparison to their genetic backgrounds (WT and amelogenin null). Attempts were made to better evaluate the consequence of increased LRAP expression on incisor growth by measuring the length of the tooth utilizing data derived from a microCT.

MicroCT Method

Level of mineralization of first molar was accessed on non-decalcified hemi-mandibles of 3 samples from P5 WT, Amelx Null, TgLRAP/WT, TgLRAP/Amelx Null respectively by micro-computed tomography (SkyScan1076; Bruker-microCT, Kontich, Belgium) with x-ray source operating settings at 100 kV and 0.1 mA. After reconstitution of images by NRecon software (Bruker-microCT), appropriate sagittal imaging planes were selected from three orthogonal sections centered at a level containing three buccal cusps

inside the reconstructed space using Data Viewer software (Bruker-microCT). Incisor length was determined by Rapidform 3D Scanning software (Seoul, Korea).

Results

Saggital images derived from the microCT show that when TgLRAP was overexpressed in the WT background, the overall appearance of the incisor in the anterior to posterior deimension appears shorter, however, no difference was detected in enamel thickness(Figure 1). Measurements of the enamel length along the long axis of the incisors, showed that the TgLRAP incisor were significantly shorter ($p<0.05$) than the WT incisor (Figure 2).

Both the AmelX Null and TgLRAP/ AmelX Null background were shorter than the WT, but longer than the TgLRAP/WT . There were no significant differences in length between AmelX Null or TgLRAP/AmelX Null incisors

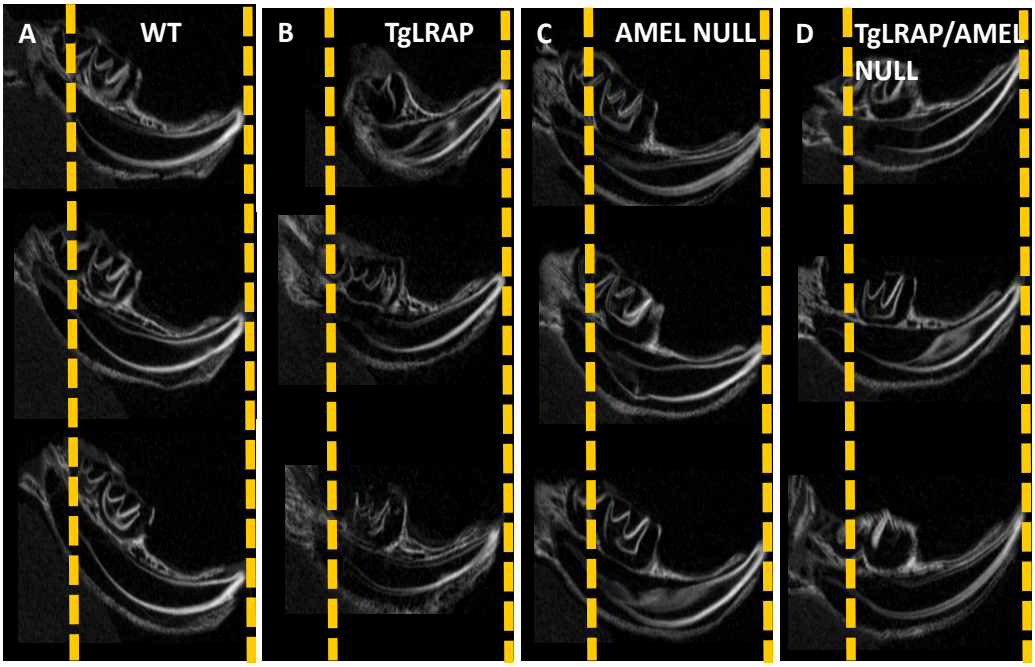


Figure 1. Saggital view of the P5 mandibular incisor. The two dashed lines on each image represent the distance between the anterior tip of the incisor to the distal surface of the first molar. The calcified labial surface of the incisor is clearly distinguishable as a white line. A thinner calcified line is present on the lingual surface as no enamel occurs on that surface.

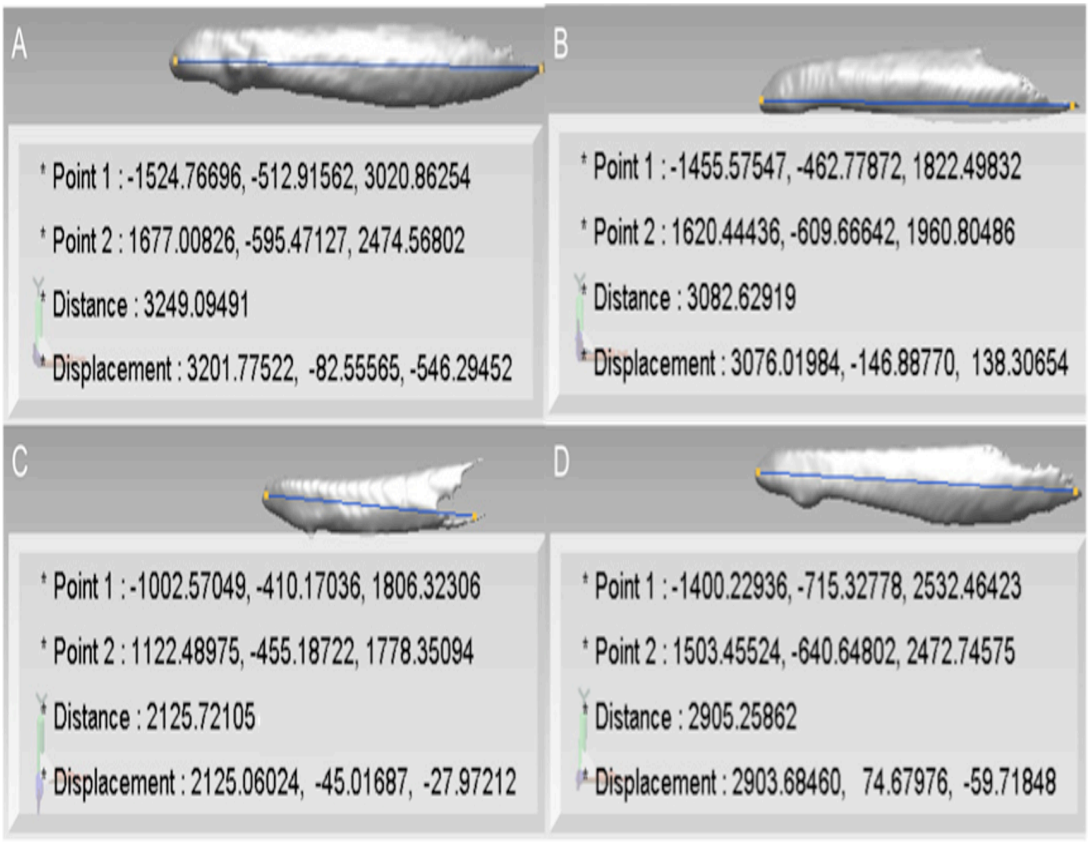
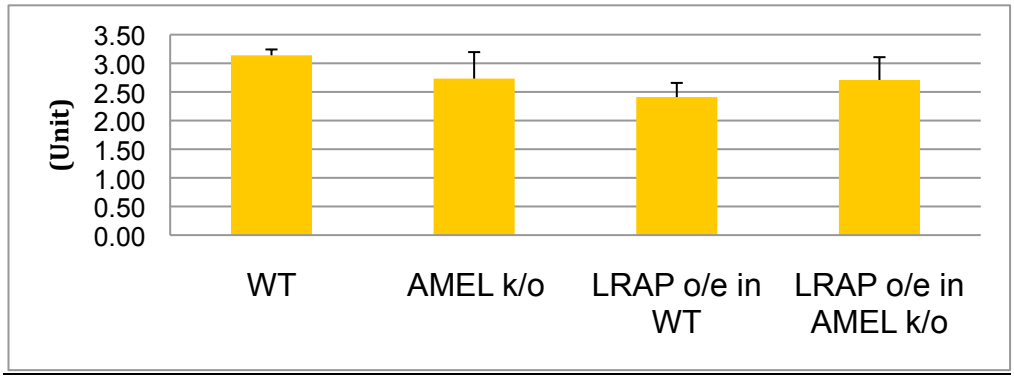


Figure 2. Representative saggital 3D long-axis incisor microCT images. (A) WT incisor with the line representing the longest distance between two calcified points of the incisor from anterior to posterior. (B) Amelogenin Null incisor (C) LRAP overexpressed in the WT background. Result of incisor length was measured by Rapidform 3D scanning software. As Rapidform could not read actual distance, measurement was recorded by unit.



Sample No	1	2	3	4
Sample	WT	AMEL k/o	LRAP o/e in WT	LRAP o/e in AMEL k/o
1	3.123	2.905	2.543	2.755
2	3.249	3.085	2.563	3.082
3	3.046	2.209	2.125	2.29
Average	3.14	2.73	2.41	2.71
STD	0.10	0.46	0.25	0.40

t-test	P-value
1 & 2	0.133
1 & 3	0.012
1 & 4	0.098
2 & 3	0.182
2 & 4	0.475
3 & 4	0.171

Table 1. Comparison of incisal length among the four murine phenotypes assayed in the microCT analysis.

Discussion

The lack of a change in enamel thickness in the TgLRAP/WT incisors as compared to WT incisors, is in agreement with previous reports (Chen et al., 2003). However, if the incisors are smaller, then a similar enamel thickness between the WT and TgLRAP mice suggests increased amelogenin synthesis. In fact in the molars, we also found increase amelogenin synthesis, with the major phenotype related to loss of polarity and ability of ameloblasts to secrete. We found downregulated SATB1, possibly this phenotype is different from that found in the molars, where secretion of enamel matrix proteins is inhibited and a thinner enamel layer is formed (Stahl et al., 2013b). Similar to the what is found in the molar model, LRAP only affects tooth formation on the WT background, indicating that the effects of LRAP overexpression require interactions with amelogenins.

In vitro, LRAP upregulates amelogenin synthesis and downregulates NOTCH, suggestion an effect on enhanced ameloblast differentiation. If indeed, LRAP overexpression promotes ameloblasts differentiation, this could explain the smaller incisor, there ameloblasts differentiate sooner, effectively reducing the length of the secretory stage.

However, it is not clear why in the molar, LRAP would inhibit cell polarization, whereas in the incisor, a similar effect appearently did not occur. In the molar, we linked the loss of polarity to reduced SATB1 protein. SATB1 protein is degraded by caspase 6, and is protected by phosphorylation of SATB1. The lack of obvious depolarization of ameloblasts in the incisor, suggests differences in phosphorylation of SATB1, or in caspase 6 activity.

The rodent incisor model is convenient for studies of enamel and dentin formation. These differences in the effect of LRAP overexpression on the molar and incisor models are interesting, as it is important to fully understand the differences in the models and how they relate to human tooth formation,

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Chapter 6. Ameloblast Lineage Cell Studies

Amelogenesis is a complex process not only because of the diverse genetic programs involved in ameloblast proliferation, morphogenesis, determination, differentiation, extracellular matrix biogenesis and biomineralization but because many of these events occur simultaneously within the developing tooth. To analyze the biochemical functions related to developmental regulation of ameloblasts, *in vitro* ameloblast lineage cell models have been deployed.

In recent work in our laboratory (unpublished) human fetal ameloblast lineage cells transfected with SV40 to create a cell line were transfected with a vector expressing both human LRAP and GFP and a control vector expressing only GFP. Fluorescence activated cell sorting FACS was utilized to isolate GFP transfected cells. The cells were collected and qPCR was carried out to analyze the relative mRNA expression of PCNA, Notch 1, and KLK4.

LRAP transfection resulted in a significant down regulation of PCNA, Notch 1 and KLK4 mRNA expression (Figure 1). These studies were difficult to repeat because of both the relatively low transfection efficiency and the high cost of FACS sorting. Therefore, I set forth to develop an alternative cell culture model to test the effects of endogenous overexpression of LRAP in ameloblast lineage cells. My goal was to the use this cellular model to more readily investigate the

signaling pathways that responded to LRAP overexpression and synthesis. The primary disadvantage of the vector previously designed in our laboratory was the small transfection rate leading to the necessity to sort cells. Therefore, I opted to design an adenovirus that could be utilized to transduce cells to assure most cells overexpressed LRAP.

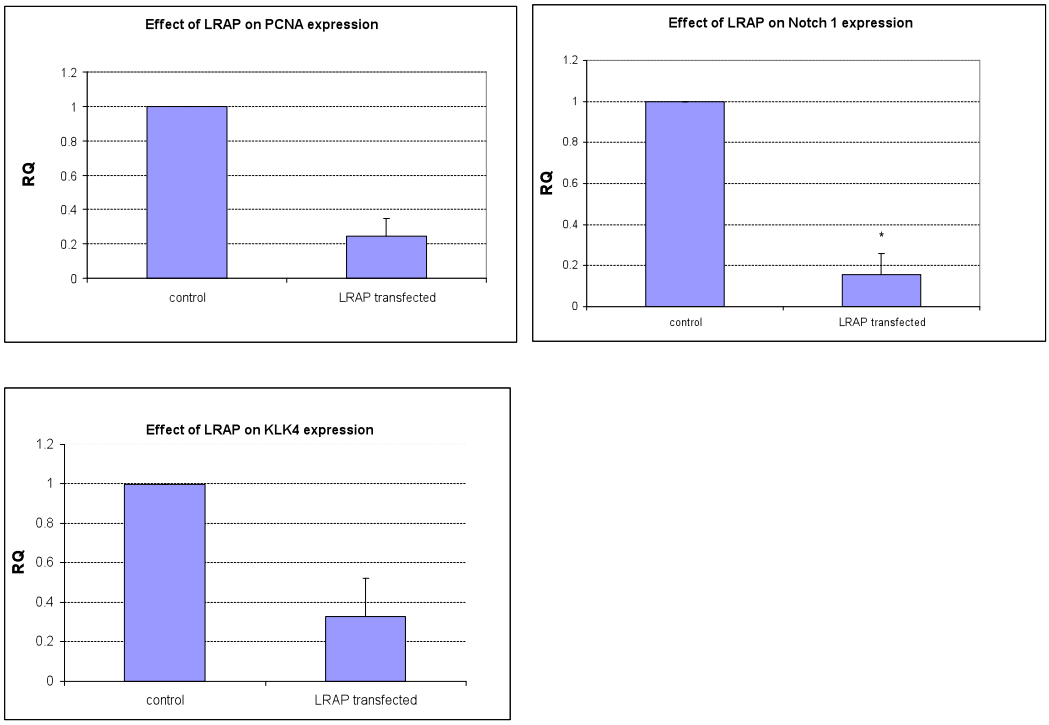

Figure 1. Real Time PCR analysis of human immortalized ameloblast lineage cells transfected with the human LRAP gene sequence show a downregulation of PCNA, Notch 1, and KLK4.

Methods

Cell culture:

We used human ameloblast lineage cells, derived from bell stage human tooth germs, and have identified these cells as derived from enamel organ epithelia, as including inner enamel epithelial cells or early and late pre-secretory cells (DenBesten *et al.*, 2005, Le *et al.*, 2007, Zhang *et al.*, 2007, Zhang *et al.*, 2006). Primary ameloblast lineage cells were isolated from 18-23 week old human fetal tooth buds, obtained through the tissue-sharing program at UCSF. The incisor, canine, and molar tissue mass was dispersed by 1 mg/ml of collagenase/dispase in PBS at 37°C for 2 hrs, and further digested with 0.05% trypsin with EDTA for 5 min at 37ºC. Ameloblast lineage cells were selectively grown in a serum free keratinocyte selective media, KGM-2 from Cambrex (Walkersville, VA, USA) supplemented with 0.05 mM calcium and penicillin/streptomycin 1x. Cells were cultured in a humidified atmosphere of 95% air and 5% $C0_2$. The primary cells were passaged only once after reaching 80% confluency.

The LS8 cell line was provided to us by the Klein Lab of UCSF, but originated from Dr. Malcolm Snead at the University of Southern California (Chen et al., 1992). Cells were plated at ~$5x10^5$ on a 10cm culture plate in Dulbeco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) with Penicillin (100 units/ml) and streptomycin (100 ug/ml). Cells were cultured in a humidified atmosphere of 95% air and 5% $C0_2$.

rtPCR and qPCR was carried out as described in chapter 3. qPCR human primers/probes to amplify Notch1, NFKB1, TGFB1, Lamp1, MMP20, KRT14, and PCNA were purchased from Applied Biosystems (Foster City, CA). Primers/probers are proprietary to Applied Biosystems, and therefore sequences are not available. Taqman master mixes for qPCR were purchased from Roche Applied Sciences (Indianapolis, IN). Murine amelogenin primers spanned amelogenin exon 5-6d: forward primer 5'CTTACCCCT TTGAAGTGGTACCA 3' Reverse 5'CCTCTGGAAGCTTGGCCAGCGACA3' and were used for rtPCR.

Adenovirus Generation:

First an entry clone was created using the pENTR™ Directional TOPO cloning kit (Invitrogen). A PCR product was generated by amplifying the bovine LRAP sequence cDNA sequence from the LRAP 19 Plasmid provided by the Gibson Laboratory at U PENN. Primers were designed to add CACC to the forward primer, resulting in primer sequences: Forward 5'CACCATGCCTCTACCACCTCATCCT3' Reverse 5'TTAATCCACTTCCTCCCGC3'. PCR was performed using a blunt ended polymerase (Stratagene). Blunt-end PCR products clone directionally at greater than 90% efficiency, with no ligase, post-PCR procedures, or restriction enzymes required, and therefore, the . PCR product was mixed with the entry plasmid in a 1 to 1 molar ratio as described by the manufacturer, and then transformed into One Shot® competent E. Coli. Six colonies were chosen for analysis and the

constructs were sequenced to confirm that the LRAP gene was cloned in the correct orientation.

Once I obtained an entry clone and verified via sequencing that it contained the LRAP gene insert in the proper direction, I performed an LR recombination reaction between the entry clone and the destination vector pAd/CMV/V5-DEST™. In brief the 25ng of LRAP Entry clone, 50ng of destination vector, RNA free water, and LR Clonase® II enzyme mix were mixed together according to manufacturers protocol and incubated for 1 hour at 25ºC. I then added 1ul of Proteinase K to the reaction and incubated at 37ºC for 10-minutes. I then transformed 2ul of the LR recombination reaction into TOP10 E *Coli* competent host and incubated in a water bath for 30 seconds at 42ºC., and plated the bacteria on ampicillin selection plates and incubated overnight at 37ºC. Six clones were selected the next morning and amplified in LB media shaking at 225 RPM and 37ºC for eight hours. A Miniprep (Qiagen, INC) to collect DNA and sequencing was done to confirm clones were positive for the LRAP gene. For two positive clones a MAXIPREP (Qiagen, INC) was performed to obtain sufficient DNA to generate a virus.

One technical issue we encountered with our viral clone is that the V5 epitope that could be utilized for detection of LRAP expressions was inadvertently not included. For the V5 epitope tag to be included in the final clone, the gene in the entry clone should not have contained a stop codon. In addition, the gene should

have been in frame with the V5 epitope tag after recombination.

Once I obtained purified plasmid DNA I digested with PacI (New England BioLabs, Inc) restriction enzyme to allow for proper replication and packaging. Digestion of the vector with Pac I allows exposure of the left and right viral ITRs and removal of the bacterial sequences. Purification of the digested plasmid was performed with a Miniprep Kit. The Pac I digested LRAP adenovirus (1 ug) was then transfected into 293A cell line using Lipofectamine 2000 (Invitrogen, Inc) to generate adenoviral stock. The virus was then collected 10 days after transfection when visible plaques had formed and most 293A cells experienced cell lysis. The culture media along with floating 293A cells to a 15ml. After three freeze/thaw cycles centrifuge tube and centrifuging at 3000 RPM for 10 minutes and aliquoting the elute at 100ul. The viral stock was amplified by adding 100ul of the crude virus to a 10ml of DMEM cell culture media and added to a 10cm cell culture plate of 293A cells. Collection of virus occurred after 72 hours as described previously. A viral titer was performed utilizing 10 fold serial dilutions of amplified virus in a 6 well culture plate of 293 cells. A control virus was made that expressed LacZ. The destination vector with a LacZ insert was provided by Invitrogen with the ViraPower™ Adenoviral Expression Sysytem (Invitrogen).

Treatment of cells with adenovirus

Multiple viral titers were tested on both primary FTAB and immortalized LS8 ameloblast lineage cells. Primary FTAB were exposed to andenovirus Multiplicity

of Infection (MOI) of 10, 20, 50, and 100. At an MOI of 50 cells began to demonstrate a phenotype characterized by blebbing after 48hrs at an MOI of 100 cells detached at the 48hr point suggesting too high a dose leading to cell death. Cells collected at an MOI of 50 were analyzed for gene expression by PCR. LS8 cells were tested at an MOI of 3, 5, 10, and 20. In general cells detached at an MOI 20 at 24 hrs, but were still viable at MOIs of 10 and below, and therefore LS8 cells at an. MOI of 10 were analyzed for gene expression by PCR analysis.

Results

Primary FTAB cells successfully upregulated the bLRAP gene after transduction

After transduction with the bovine LRAP adenovirus the overexpressed gene could be detected by PCR with no LRAP detected in cells that were not transduced (Figure 2). FTAB cells formed colonies of cobblestone shaped cells which is typical for these epithelia derived cells (Figure 3).

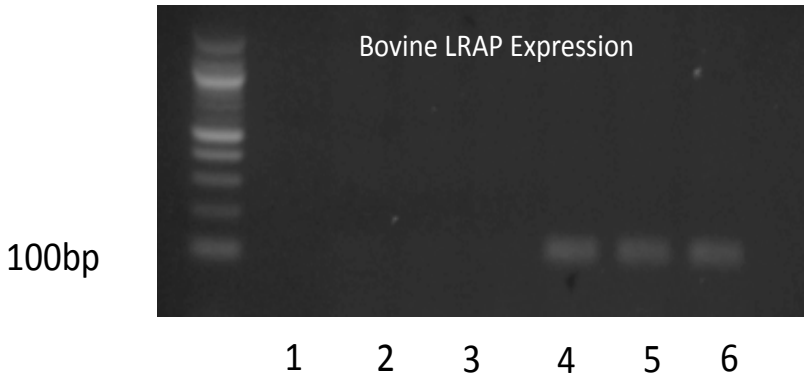

Figure 2. Bovine LRAP expression after transduction of primary FTAB cells with LRAP adenovirus. Lanes 1-3 were not transduced with the virus. Lanes 4-6 were transduced with LRAP and show expression of the LRAP gene indicating successful expression of the gene into the cells.

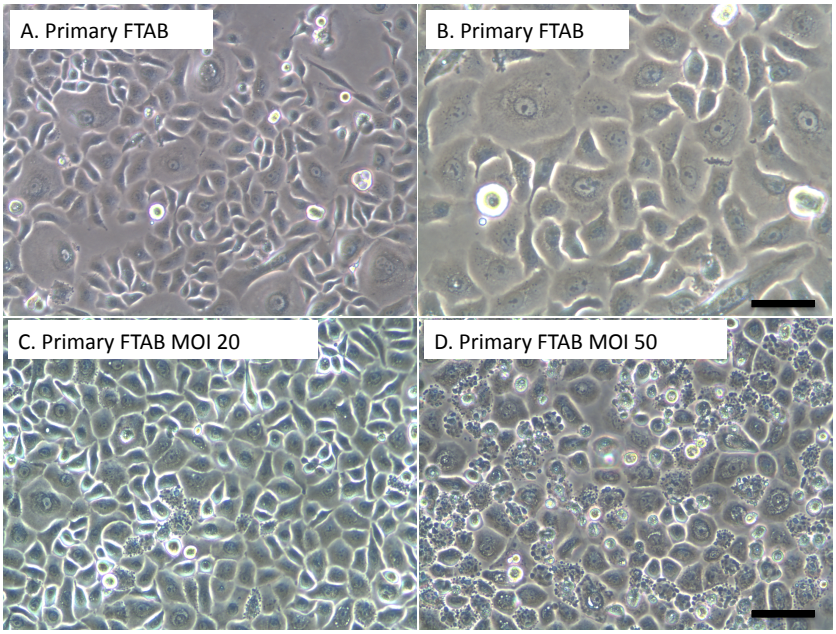

Figure 3. Morphology of cultured primary human fetal tooth ameloblasts after transduction with LRAP. A) Typical cellular phenotype of monolayer cultured cells in keratinocyte growth medium was a combination of small cobblestone shaped cells and large rounded cells. B) represents an enlarged phase contrast image. C) treatment of cells with LRAP adenovius at an MOI of 20. After 48 hrs no changes were not to cell morphology. D) Treatment with an MOI of 50 led to pronounced blebbing on the cell surface at 48 hrs. Scale bar 50 μm for A,C &D; 30 μm for B.

Nf-Kappa beta 1 and Notch 1 were downregulated

LRAP tranduced primary human ameloblast cells showed a 4.2 fold ($p<0.05$) decrease in Nf-kappa beta 1, and a 2.4 fold downregulation of Notch1 expression (Table I). There were no significant effects on expression of amelogenin, MMP-20, PCNA, LAMP1 or KRT14.

Table I. qPCR analysis of genes related to enamel development

GENE		Fold Change
MMP20	⇑	1.1
PCNA	⇑	1.2
TGFβ1	⇑	1.2
Notch1	⇓	2.4
NFKB1	⇓	4.2
Lamp1	⇑	1.2
KRT14	⇑	1.4

LRAP upregulated full length amelogenin in the LS8 cell line

LS8 cells present with a morphology similar to a fibroblast as opposed to the cobblestone phenotype of individual human FTAB cells (Figure 4). Cells were transduced with LRAP adenovirus for 48 hours as by 72 hrs significant cell detachment and cell death occurred (Figure 2). When the LS8 murine derived ameloblast lineage cells were transduced with the LRAP adenovirus an increase in amelogenins (containing exon 6) was detected as a distinct band on the acrylamide gel at just over 500bp in size (Figure 5), but this result could not later be repeated.

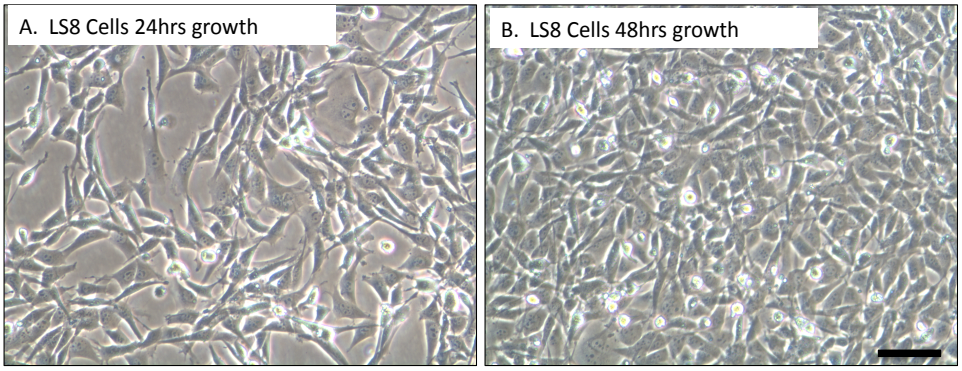

Figure 4. LS8 Ameloblast lineage cells form multiple layers 48hrs after plating. (A) P11 LS8 cells plated on a 10cm plastic plate at 24hrs after transduction with LRAP Adenovirus wit an MOI of 10 (B) at 48hrs confluency is reached and cells began to form layers typical of LS8 cells at this stage. Scale bar 50 μm.

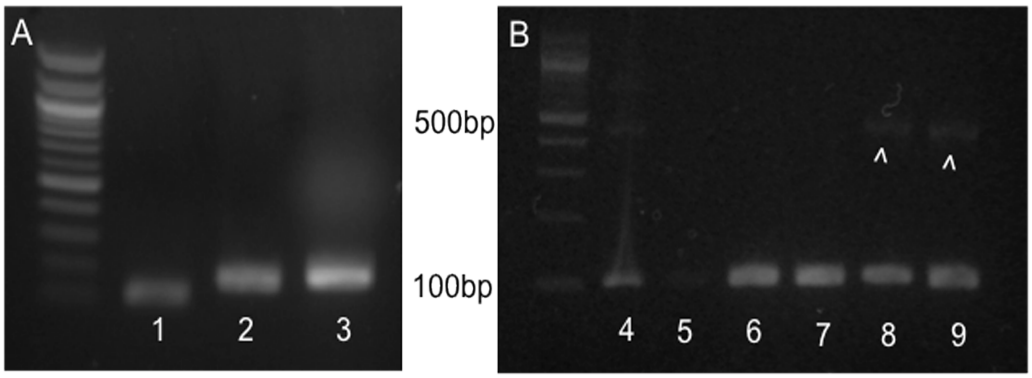

Figure 5. A) Murine amelogenin primers could detect bovine LRAP . in lane 1 primers were designed using the bovine LRAP sequence from amelogenin exon 2 to exon 6d and tested against a bovine plasmid containing the bovine LRAP cDNA sequence. In lanes 2&3 amelogenin primers were generated using the murine amelogenin sequence extending from exon 2 to 6d (lane 2) and exon 2 to 7 (lane 3). The murine primers reacted with the bLRAP cDNA. B) LRAP overexpression in LS8 cells increased expression of full length amelogenin. The murine ameloblast lineage cell line was transduced with bovine LRAP adenovirus. Controls (lanes 4&5) did not express a band that would be suggestive of full length amelogenin, but did express a weak band that would be indicative of LRAP using primers that started with amelogenin exon 5 and ended with amelogenin exon 6d. LS8 cells (lanes 6&7) transduced with MOI of 1 & 3 respectively did not show evidence of full length amelogenin expression, however, a band at ~100bp similar in size to LRAP was present and expressed more intensely than equivalent bands present in the controls. When cells were treated with an MOI of 5&10 respectively a weak bands at ~500bp were seen and these bands would coincide with the expected size of full length amelogenin.

LS8 cell growth was not sustained in keratinocyte growth medium

Efforts to grow the LS8 cells in keratinocyte growth medium, as is used for FTAB cells, led to cells that failed to proliferate after 48 hours (Figure 6). This may suggest the LS8 ameloblast lineage cell is not a true distinct epithelial cell line

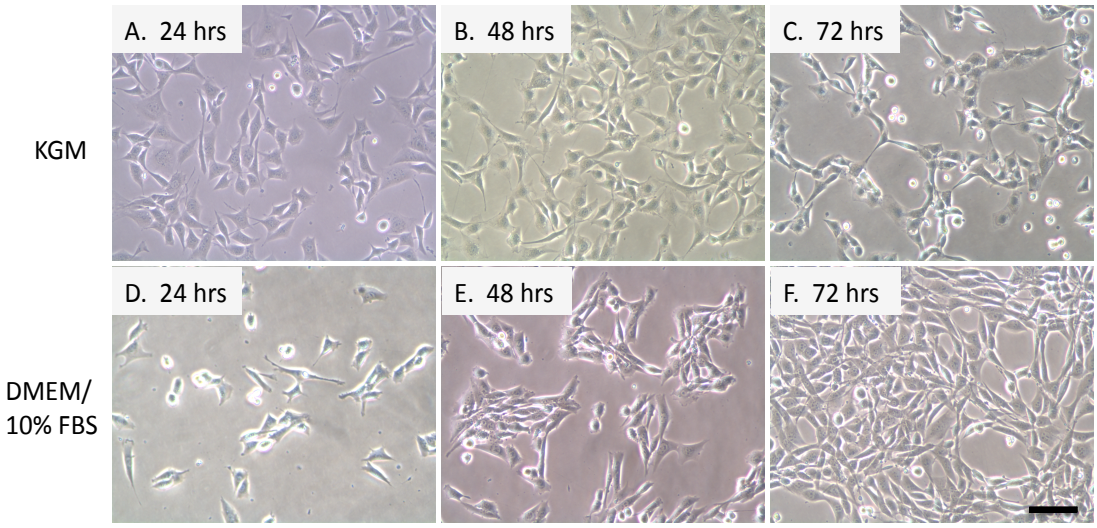

Figure 6. Proliferation of LS8 cells in serum containing growth media vs. keratinocyte growth medium (KGM). LS8 cells were plated at a density of $1x10^4$ on a 10cm plastic dish. In DMEM supplemented with 10% FBS (A-C) cells formed typical colonies, which changed little at 48 hrs and appeared to cease growing by 72 hrs. In KGM the cells showed initial signs of clustering at 24 hrs, which grew to distinct colonies at 48 hrs and colonies in markedly in size at 72 hrs. Scale bar 50 μm.

Discussion

In these studies, I explored the use of ameloblast lineage cells to determine how LRAP alters gene expression. Somewhat unexpectedly, over expression of LRAP cDNA did not significantly upregulate amelogenin expression, similar to what we found *in vivo* when LRAP was overexpressed in WT mice (see Chapter 3). This is likely due to the fact that both of these cell types had no or minimal amelogenin gene expression.

The factors that control amelogenin gene expression remain unknown and the results of these *in vitro* studies suggest that when LRAP cDNA is transfected into ameloblast lineage cells, only low levels of LRAP protein or other amelogenins are synthesized. These results differ from those of Le et al (Le et al., 2007), who found that when exogenous LRAP protein was added to FTAB, that both amelogenin protein and LAMP1 were increased. Therefore, it seems that amelogenin synthesis may be regulated in part by amelogenins present in the extracellular matrix, possibly mediated by the LAMP1 cell surface receptor.

However, similar to the studies by Le et al using exogenous LRAP, we found evidence of an upregulation of Notch signaling in both the lentivirus transfected primary ameloblast lineage cells (FTAB) and the SV40 immortalized ameloblast lineage cells. Notch signaling is an evolutionarily conserved signaling pathways in the development of multi-cellular organisms (Kopan and Ilagan, 2009). Its

temporal-spatial expression effects can specify diverse cellular events, including proliferation, differentiation, apoptosis, and stem cell maintenance. Notch1 has been shown to function in the maintenance of undifferentiated epithelial stem cells (Harada and Ohshima, 2004), and has been shown to regulate tooth morphogenesis and ameloblast differentiation (Mitsiadis et al., 2010).

NFkB1 was most dramatically affected, showing a 4.5 fold downregulation when FTAB were transfected with the LRAP lentivirus. NFkB1is a transcription factor that mediates the survival response of many signals by inhibiting p53-dependent apoptosis and up-regulating anti-apoptotic members of the Bcl-2 family, and caspase inhibitors such as XIAP, and FLIP (Busca et al., 2009). Down regulation of NFkB1 would suggest a possible increase in apoptosis that may relate to increased terminal differentiation of the ameloblast lineage cells.

It is interesting that Notch1 and NFkB1 share many common features and both regulate similar target genes such as Hes-1 (Ang and Tergaonkar, 2007). Downregulated Notch-1 expression is observed in mice with reduced NFkB activity and is believed to contribute to incisor development, suggesting a functional cross talk between these pathways (Ohazama et al., 2004). The evolutionarily conserved cellular characteristics of apoptosis are defined by events that occur during the final stages of death, including cell contraction, dynamic membrane blebbing and DNA fragmentation (Coleman et al., 2001). The distinct cellular blebbing seen on the cytoplasm of LRAP transduced FTAB

cells may further be a sign of apoptosis contractile force generated by actin–myosin cytoskeletal structures are hypothesized to lead to the formation of membrane blebs (Mills et al., 1998).

We found PCNA downregulated in immortalized ameloblast lineage cells transfected with LRAP, but not in the primary cells. It may be the effects of LRAP in downregulating PCNA expression, which is a marker of cell proliferation, is only evident in the more rapidly proliferating SV40 transfected cells. A downregulation of cell proliferation is also consistent with cell differentiation.

In vitro cell culture models serve as an important tool for examining the genes that are of importance for the maintenance of the cell-matrix interactions, cell-signaling, regulation of cell metabolism, and control of the mineralization process in the early developmental stages. One of the challenges in developing cell culture models to study amelogenesis is the inability to promote cell differentiation, including amelogenin synthesis. In these studies we used a number of cell culture models to investigate the role or LRAP on gene expression. However, I realize that *in vitro* experiments using isolated ameloblast-like cells, must be viewed with a great deal of caution. Primary cultured ameloblasts are advantageous because they are reported to keep differentiated character *in vitro*, including the expressions of ameloblast specific genes and the potential to form calcified nodules, but as with other primary cells have a restricted potential of cellular proliferation (Kukita et al., 1992; Li et al.,

1998; Nakata et al., 2003). Although the *in vitro* portion of my work is not as robust as the *in vivo* work it does further support the hypothesis that LRAP functions to promote and regulate ameloblast differentiation.

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Chapter 7. Conclusions

This dissertation was primarily focused on the analysis of tooth formation in a murine model when the alternatively spliced variant, called leucine-rich amelogenin peptide is overexpressed. Amelogenesis is a very complex process resulting in a highly specialized tissue, and overall my findings suggest that LRAP plays a critical role in regulating amelogenesis.

Initially, unsure if LRAP overexpression would result in a phenotype in the early developmental stages of amelogenesis my first goal in this project was to explore the effects of LRAP overexpression on tooth formation at different time points in early postnatal development. I felt that the ideal model for exploring the effects of LRAP overexpression was the crossing of a TgLRAP mouse with an amelogenin null mouse, as this would eliminate all other amelogenin isoforms except LRAP.

However, surprisingly my histological analyses did not reveal an obvious phenotype in this model when compared to the amelogenin null mouse alone. The enamel matrix thickness was significantly reduced in both models compared to the WT with no discernable difference between them. However, even more surprising was that when LRAP was overexpressed on the WT background, that a significant change occurred.

The ameloblasts in the P5 TgLRAP/AMELX null and the AMELX Null amelogenin null mouse molars were primarily secretory stage cells, and demonstrated the typical long columnar appearance of the cells in both the models, with neither showing obvious increases in secretory vesicle formation. Secretory ameloblasts in both models lacked normal Tomes' processes. Our microCT of mandibles demonstrated no obvious difference in the incisor or molar of the mineralized tissue formation between these two models.

In contrast to the AmelX null model when LRAP was overexpressed in a WT background a clear histological phenotype was observed by the secretory stage of development. Ameloblasts demonstrated a disorganized pattern with an extensive increase in amelogenins in the cell. To verify that the overexpressed amelogenin seen was not transgenic LRAP we first looked at amelogenin expression in pre-secretory ameloblasts and found a significant upregulation of the signal for the full length amelogenin gene. On the protein level we checked amelogenin expression with antibodies specific for exon4 and exons8&9 and found they were both increased. Taken together this evidence led to my conclusion that LRAP had a specific affect in regulating early stage ameloblast differentiation. Further the findings suggested that LRAP played a role in controlling the expression of multiple spliced variants of amelogenin. When only M180 was overexpressed in the WT background we found no ameloblast phenotype, further indicating the unique function of secreted LRAP in altering ameloblast morphology.

After determining the extent of the phenotype in the different stages of ameloblast differentiation my next goal was to look specifically at potential markers of ameloblast differentiation related to enamel matrix formation. Stage-specific expression of ameloblast-specific genes is controlled by differential expression of transcription factors, adhesion molecules and growth factors.

Not only was the amelogenin message upregulated in the preameloblasts but other members of the proline-glutamine-rich subfamily, which comprise the enamel matrix genes were also upregulated. Among these were enamelin and ameloblastin that are associated with the mineralization front which is a place of continual protein turnover where the enamel ribbons, comprised initially of amorphous calcium phosphate grow in length. An earlier increase in these genes would further suggest earlier enamel matrix formation and mineralization, and this was supported by our findings in chapter? where our trichrome staining indicates earlier hydrolysis of the enamel matrix. As amelogenesis progresses through a maturation stage, specialized enzymes are secreted from ameloblasts to digest enamel matrix proteins. If earlier formation of the enamel matrix was indeed occurring it would be expected that increased synthesis of the proteolytic enzyme MMP20 would occur and we did show an abundant upregulation of the gene signal for MMP20 in presecretory ameloblasts as well as potential increased protein expression of MMP20.

Although the mechanistic pathway(s) through which LRAP alters ameloblast differentiation are yet to be defined, the down regulation of SATB1 provides novel

insight into LRAPs potential to regulate ameloblast differentiation *in vivo*. In addition to the role as a globlal chromatin organizer in progenitor cells SATB1 is also a master transcription regulator and SATB1 downregulation may have altered the expression of numerous genes and pathways critical to ameloblast differentiation. This is the first report of SATB1 involvement in enamel organ formation.

Another protein for which we found a different result than what is currently reported in the literature is LAMP1. LAMPs are necessary for the structural integrity of the lysosomal membrane; helping to keep enzymes separated from the cell cytoplasm. LAMPs are also found on the plasma membranes of ameloblasts and the membranes of endocytotic vesicles, and have been shown to be a binding partner to LRAP. LAMP1 has a role in endocytosing LRAP and other amelogenins. *In vitro*, as the addition of exogenous LRAP was increased, LAMP1 was also increased (Le et al., 2007). However, when LRAP was endogenously overexpresed in our *in vivo* model there was no increase in LAMP1. However, in thinking further, it seems that a possible reason for this lack of an effect on LAMP1 is the observation that amelogenins were not secreted from the LRAP overexpressor ameloblasts. Without additional extracellular amelogenins, LAMP1 would not be required for resorption of amelogenin fragments, and would therefore not be upregulated in this model.

One surprising finding in this study was the high level of exon4 protein expression in the TgLRAP model. This upregulation of amelogenins transcripts containing exon 4 may indicate a role for LRAP on the control of alternative splicing of amelogenins. I further

examined the expression pattern and possible function of exon4 in amelogenesis. I found that amelogenin protein variants derived from exon4 containing transcripts were principally expressed in the early maturation enamel matrix. This led to my hypothesis that exon4 may have a function in mineralization of the enamel matrix which primarily occurs in this stage. This hypothesis was supported by a bioinformatics analysis that does indicate that exon for has enhance amelogenin binding. We propose that this binding inhibits crystal growth at the late transition stage when proteinase synthesis is dramatically upregulated. Inhibition of apatite growth by exon 4 peptide containing amelogenins may then allow the proteins to leave the apatite space before mineralization proceeds.

Although most of the studies in this thesis were carried out using *in vivo* experimental models, some experimental approaches were performed *in vitro*. To gain further insights into the cascade of events and the cellular mechanisms that are involved in amelogenesis, I used an adenovirus to endogenously overexpress LRAP in ameloblast lineage cells. Down regulation of biological signals such as Notch1 and NFKß1, which suggests earlier terminal differentiation of the cells supports our finding in the *in vivo* model that LRAP play an important role in modulating ameloblast differentiation. Ameloblast lineage cells are only able to proliferate in the pre-ameloblast stage of development so it proved difficult to reproduce the *in vivo* findings as a whole *in vitro*. However, I was able to show an upregulation of the full length amelogenin message in the LS8 cell line when LRAP was overexpressed.

The exact function of LRAP in amelogenesis remains unclear, but the findings of these studies strongly support the hypothesis that LRAP is a regulator of ameloblast differentiation and for the first time, provides *in vivo* evidence of this function.

In summary, these studies were the first to report the effects of *in vivo* overexpression of alternatively spliced amelogenin LRAP on ameloblast differentiation. The findings in this thesis provide novel insights into the regulatory mechanisms controlling ameloblast differentiation and dental enamel formation. This knowledge will benefit future research aimed at tooth regeneration and using amelogenins to bioengineer enamel. Future studies, related to this work will focus further on candidate signaling pathways, which may be downstream of LRAP.

References:

Le TQ, Zhang Y, Li W, Denbesten PK (2007). The effect of LRAP on enamel organ epithelial cell differentiation. *J Dent Res* 86(11)*:*1095-1099.

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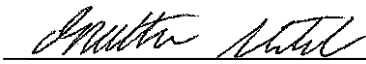

31 Aug 2013

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