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The analysis of ameloblastin and a human tooth germ cell culture system



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# The Analysis of Ameloblastin and a Human Tooth Germ Cell Culture System

by

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## Dedication and Acknowledgements

I would like to dedicate my dissertation to my loving wife Phyllis. She became my wife during the project, and supported me along the way never questioning the long hours and the hard work. Her late nights in the lab either helping me or just being by my side meant so much to me. Thank you Phyllis for being you. I love you.

I would like to acknowledge everyone in the laboratory for all their help with the work on this project. Science is a team project, and it could not have been accomplished without help from the whole team. Thank you all.

I would also like to thank my parents for all the understanding they have given me over the years. I am so fortunate to have parents the caliber of mine.

What a long strange trip it's been.

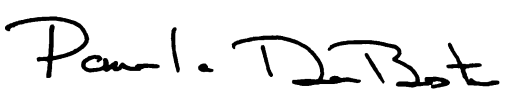
# ABSTRACT

The analysis of Ameloblastin and a Human Tooth germ Cell Culture System

By

Darren Machule

The protein ameloblastin is the second most common tooth enamel protein, yet there is little data on this protein or gene sequence. The bovine ameloblastin gene sequence was determined by library screening and PCR amplification of alternatively spliced mRNAs. This sequence information resulted in the identification of a new splice variant with unique predicted phosphorylation sites that were either spliced in or spliced out. Functional analysis studies were initiated using immunoperturbation of a mouse tooth germ organ culture and an antibody against the protein ameloblastin. These immunoperturbation experiments of the cultured tooth germs established a possible function of ameloblastin as a negative suppressor of enamel formation. In order to further understand the ameloblastin protein, a novel "ameloblast-like" primary cell culture was established and characterized. The cell culture system was used to identify two different cell types, cobble stone shaped epithelial cells (CABS) and spindle shaped cells (SPABS). The cells were characterized by identifying enamel specific mRNA expression and protein secretion using PCR, immunohistochemistry and Western Blot. Both cell types showed similar "ameloblast-like" characteristics in relation to protein and mRNA expression. Immunohistochemical analysis of amelogenin mRNA expression, showed a nuclear staining pattern in the CAB cells with an anti-amelogenin antibody, where the SPABS showed cytoplasmic staining. Another difference was in the reliance of CAB cells on calcium, which was similar to that found in keratinocytes in culture. The localization pattern and functional significance of these results will be analyzed in future studies. These studies will also include the analyzing the predicted phosphorylation pattern of ameloblastin through cell culture. Although further studies are necessary to confirm the possible function of ameloblastin, and it's role in ameloblast function, the characterization of ameloblastin splice variants, and the development of a primary cell ameloblast cell culture system, described in this dissertation, are a major advance in our understanding of the biology of tooth enamel formation The results of this dissertation project lead to further advances on the ameloblastin protein, and show major advances in the field of tooth development with the establishment of a novel cell culture system.



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## Chapter 1 General Introduction

The general introduction briefly discusses the hypothesis for each chapter of this dissertation. It is discussed in relevance to the current literature setting a stage for the preceding scientific problems, and rationales for the experimental approach. In part two of this chapter the background, significance, and rationale are given to enable the reader to understand the complex problems associated with this field.

Ameloblastin is the second most abundant tooth enamel matrix protein, yet little is known about the function(s) of this protein. Initial identification of this protein occurred through three laboratories who independently cloned either rat or porcine ameloblastin [2-4]. These ameloblastin cDNA sequences showed a high degree of homology, leading to the hypothesis that this homology would remain in the bovine species. The initial focus of the dissertation experiments was to characterize ameloblastin in an animal model large enough (bovine) to harvest sufficient amounts of enamel matrix to allow parallel molecular and protein biochemical analysis of this interesting protein.

Understanding the role of ameloblastin in tooth formation is critical for future discoveries in the regulation of enamel formation and biomineralization. Fully formed enamel is a unique acellular matrix and a model for the generation of unique biomaterials. By understanding the roles of specific proteins, perhaps new biomaterials can be created that more naturally mimic the native tooth structure leading to longer lasting restorations. The hypothesis was that the highly conserved ameloblastin protein has a role in tooth development. To elucidate a mechanism of ameloblastin function during tooth

development, a tooth organ culture system and a function-perturbing antibody to ameloblastin was used (chapter 3). The tooth organ culture system has proven to be a valuable tool in the study of epithelial-mesenchymal interactions during mandibular morphogenesis. Although most of the studies using tooth organ culture have been done with antisense molecules, Mitsiadis and coworkers have shown that an antibody to the differentiating factor, midkine, worked to immunoperturb the tooth organ culture system [5]. In this project an antibody against a specific fragment(s) of ameloblastin was used in a mouse tooth germ organ culture to elucidate ameloblastin's function.

To further examine the hypothesized role of ameloblastin in tooth development, the next goal was to purify native ameloblastin from bovine enamel extracts (a readily available protein source in the lab). The purified protein was to be utilized in three ways. First, the literature supported the post-translational modification of ameloblastin, though a full-length post-translationally modified protein had not been isolated and sequenced. Second, was to determine whether matrixmetallo-proteinase-20 (MMP-20) led to the rapid degradation of ameloblastin that had been reported in the literature [6, 7]. Third, was to further decipher the mechanisms of ameloblastin function by the addition of this purified protein into the tooth germ culture system (as shown in chapter 3). The addition of ameloblastin back into the organ culture system with and without anti-ameloblastin antibody, would act as a control with the antibody experiments, and as an overexpression of ameloblastin to further analyze function. The purification project taught me key protein purification and identification methodology, however, bovine serum albumin (BSA) a major component of enamel matrix [8] , could not be separated from the full-

length ameloblastin in these experiments. However, these efforts in this project were helpful in characterizing MMP-20, leading to a co-authorship on a publication, which identified the functional domain of this enzyme [9]. The data regarding the purification of BSA is not presented in this dissertation.

The availability of ameloblastin protein remained important to the hypothesis that ameloblastin negatively regulated enamel formation by binding calcium and functioning as a calcium sink (chapter 3). A limited, but successful experience in the lab developing a porcine primary and immortalized tooth germ derived cell culture system had been achieved [10, 11]. However, the primary culture system, along with others reported in the literature resulted in either a mixed culture system, or with more selection, the inability to consistently pass the enamel protein producing epithelial cells. Although, the immortalized porcine "ameloblast like cells" (PABSO-e) studied in the lab were stable, they did not produce ameloblastin. To produce post-translationally modified ameloblastin a tooth-specific cell culture system from an earlier stage of enamel formation was necessary. A colleague in a different lab (Dr. Jim Simmer) had attempted to express the post-translational modification of another enamel protein, amelogenin, which although expressed was not post-translationally modified in a non-tooth derived cells (personal communication), further emphasizing this reality.

It was hypothesized that the developing tooth germ cell types could be cultured and used for analysis of tooth development and biomineralization. The task of establishing a new cell culture system was difficult, but necessary to move the entire field forward. The

subhypothesis was that a primary culture of the readily available porcine tooth germs could be established that maintain some of the characteristics of ameloblasts. The goal was to then to use the porcine tissue to develop a core technique, and carry this to the human counterpart. The long-term scientific vision was two-fold. First, to use the human engineered protein, with the correct post-translational modifications, to stimulate periodontal tissue regeneration and cementum reattachment (a crude mixture of enamel extracts, EMDOGAIN, has been used to do this). However, the active protein component is not known. Second, to use the human cells in tissue engineering studies for the analysis of tooth regeneration and development. Tooth regeneration is proving to be a very exciting field. Tooth regeneration will require many more studies. The ability to grow human "ameloblast like" cells will be crucial to those studies, including the analysis of the individual cell types within this cell culture population.

By utilizing the techniques learned through the analysis of porcine tissue, and the idea that tooth germs are derived from buccal mucosal keratinocytes during early development, a new hypothesis was developed. The hypothesis was that a human ameloblast-like cell culture could be established by controlling the amount of calcium in the media, similar to that found in keratinoyctes. These experiments were further carried out in human tooth germ derived cells to separate a mixed cell culture population shown in the porcine culture experiments, and to analyze serum and calcium effects on these cells. A subhypothesis from these experiments was that two human tooth germ derived phenotypically different cells (as observed in culture) produce "ameloblast-like" characteristics.

The individual hypotheses are tested in chapters 2-6 of this dissertation. Included at the beginning of each chapter is a further introduction of the literature specific to the scientific problem associated with that chapter. Background relevant to the general hypothesis that ameloblastin is important for the development and biomineralization of the tooth is given in the following paragraphs.

**Background, Significance, and Rationale**

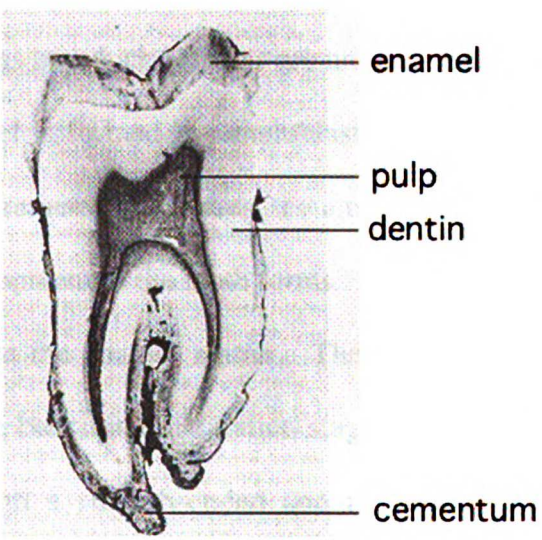


**Figure 1.** Developed tooth [1].

Tissue engineering is an emerging field with the prospect of growing organs, including teeth. Tissue engineering is no longer a dream, but a possible reality. In order to control the engineering of this highly mineralized tissue, the basic principles of tooth development and mineralization must be elucidated. The process of tooth development is a unique biological process that involves formation of three different mineralized tissues: enamel, dentin and cementum (see Figure 1). The outermost layer of the tooth crown is the mineralized enamel layer, produced by ameloblasts. The dentin layer, produced by odontoblasts, lies beneath and is in intimate

contact with the enamel layer at the dentinoenamel junction, and forms the bulk of the tooth. Cementum produced by cementoblasts is continuous with the dentin where the crown of the tooth ends, and lines the outer root surfaces. Tooth development entails a sequence of events with epithelial (ameloblasts) and mesenchymal (odontoblasts) interactions. These events occur through reciprocal induction where internal dental epithelium stimulates the differentiation of adjacent dental papilla cells to differentiate into dentin-producing odontoblasts. The dentin in turn stimulates the internal dental epithelium cells to further differentiate into the enamel-producing ameloblasts [1].

Dentin and cementum share many characteristics with bone, whereas enamel and the process by which it forms are unique. Bone and dentin are continuously forming, while enamel is formed by ameloblasts only during tooth development. Second, unlike the other mineralized tissues, the ameloblasts go through many different temporal stages of development as the tooth forms. Preameloblasts give rise to secretory ameloblasts, which secrete the enamel matrix. The secretory ameloblasts then enter a transitional stage before becoming maturation stage ameloblasts. The maturation stage ameloblasts cycle through a smooth-ended and ruffle-ended appearance while the organic matrix is degraded and enamel mineralizes [12]. These maturation cycles give rise to a dramatic change in localized pH within the enamel matrix. Eventually the ameloblasts are stimulated to enter apoptosis [13-15], and the tooth erupts with a highly mineralized acellular enamel matrix. What controls the differentiation of the ameloblasts is still unknown.

The process by which the enamel matrix mineralizes is highly regulated. Enamel crystals are almost ten times larger than those formed by the other mineralized tissues [1]. These crystals are formed as proteins secreted from the ameloblasts give rise to the extracellular matrix (ECM). The enamel matrix forms the entire enamel thickness before ameloblasts enter the maturation stage [1]. The known proteins involved in this matrix production include amelogenin (> 90% of the secretory enamel matrix), the non-amelogenins (enamelin, tuftelin, and ameloblastin), and two proteases (enamelysin [MMP-20] and kalikrein 4 (aka enamel matrix serine proteinase [EMSP1]) [16]. The emerging theories of enamel mineralization include possible interactions of the splice variants of amelogenins and non-amelogenins, as well as specific functions of the degradation products of these amelogenin and non-amelogenin proteins. These proteins and peptides all interact in an orchestrated process leading to the mineralization of teeth. How all these enamel protein interactions occur is still unknown, providing a challenging problem in the study of enamel mineralization.

Extensive research concerning the function of amelogenin has led to one of the current theories that tooth mineralization involves a spherical sub-unit formation around developing enamel crystals controlling growth in an ordered fashion [17]. This self-assembly of amelogenin has also been shown through the yeast two-hybrid system, further substantiating the current theory that amelogenin aggregates play a role in biomineralization. This same system, however, revealed that ameloblastin does not self-assemble nor does it interact with amelogenin or tuftelin [18]. Thus, the function of

ameloblastin and how it contributes to the process of tooth development is still largely unknown.

The highly organic matrix that is initially deposited by the ameloblasts during the secretory phase of enamel formation is removed during maturation, resulting in a final matrix that is 96% mineral, 4% organic material and water (weight %) after mineralization is complete [1]. Ameloblastin has been predicted to be about 5-10% of the early secretory enamel based on the quantity of mRNA relative to amelogenin [1, 3], making it the most abundant matrix protein second to amelogenin.

Ameloblastin cDNA has been cloned by five different groups, thereby giving rise to three names for the same protein: ameloblastin, amelin, and sheathlin [2-4, 19, 20]. However, fragments of this protein have been studied for years with the more stable N-terminus products localized to the sheath space, hence the name "sheath protein" and sheathlin [21]. This protein is referred to as ameloblastin in this report because this name was designated by the group who first published the full-length clone [2].

Studies of these ameloblastin degradation products have led to some insight into the possible function of ameloblastin, but have generated more questions than answers. Peptide antibodies to different parts of the ameloblastin protein have been used to study enamel formation. For example, the C-terminus of ameloblastin has been localized to the outer enamel matrix, whereas the smaller N-terminus fragments localize to the enamel matrix sheath space throughout the entire matrix [6, 21, 22]. Although a 62- and a 70-

kDa fragment are found in the enamel epithelium, only smaller products are found in the enamel matrix [23]. These studies suggest that following secretion, ameloblastin is hydrolyzed, but the investigators have not addressed questions to the possible function of ameloblastin in enamel biomineralization. Some difficulties that have been encountered in studying ameloblastin include not having access to large enough amounts of protein for further studies and the uncertainty regarding the nature of post-translational modifications required in a recombinant protein.

Ameloblastin has been much better characterized in enamel than in dentin or cementum. Immunohistochemical and *in situ* hybridization studies have shown ameloblastin to be expressed by both preodontoblasts, cementoblasts [24-26], as well as ameloblasts, from their presecretory stage to maturation [23]. This suggests that ameloblastin may be involved in the formation of all three mineralized tissues of teeth (enamel, dentin, and cementum). Although the localization of ameloblastin in these different tissues is very intriguing and important to understand in terms of possible functions, the focus of this report is to analyze the ameloblastin gene and protein (through immunoperturbation) in relation to possible functions in the process of enamel biomineralization and tooth development. Future studies will include analysis of dentin and cementum formation, as well as further analysis of enamel mineralization.

Two intriguing questions about ameloblastin that have been debated in the literature include its possible post-translational modifications. The apparent molecular mass of ameloblastin through sequence analysis is 45-kDa [2]. This, however, does not

correspond to the molecular masses found for this native protein. The molecular mass of the parent protein has been estimated to be 62-70 kDa by SDS-PAGE [23]. Although SDS-PAGE is known to give aberrant results in its estimation, this does not account for the large discrepancy. To account for this molecular weight difference, Uchida and coworkers have suggested that ameloblastin is a heavily O-glycosylated protein [6]. Another suggestion is that the transient sulfated protein identified by Smith and coworkers is ameloblastin [27]. The sulfated enamel proteins seem to match ameloblastin with the correct molecular masses, rapid degradation, and potential glycosylation; however, the sulphated enamel protein has been suggested to be N-glycosylated [27]. Whether or not ameloblastin is glycosylated and/or sulphated, and if so, at what sites, remains to be determined.

Another interesting question regarding ameloblastin is in its ability to act as a signaling molecule. Ameloblastin mRNA is found from early presecretory to late maturation stage in ameloblasts. Other enamel proteins, such as tuftelin, are seen earlier than ameloblastin [28]. While amelogenin and enamelin are also present in ameloblasts, unlike ameloblastin they are not found in late maturation stage [29, 30]. The timing of ameloblastin expression led, Lee and coworkers to hypothesize that ameloblastin may have an intracellular signaling role since ameloblastin is found in ameloblasts throughout late maturation [23].

Sequence data suggests that ameloblastin acts as a signaling molecule [2, 3]. Both rat and mouse ameloblastin contain two cell-binding sequences: DGEA (an integrin recognition

sequence) and VTKG (a thrombospondin-like cell adhesion domain) [3]. Bovine ameloblastin does not have either of the cell-binding sequences found in mouse/rat, but contains one known cell-binding sequence (RGD) [19]. The porcine and human ameloblastin cDNA contain no known cell-binding sequences. Localization studies by Uchida and coworkers [6] showed an antipeptide antibody (NKVHOPOVHNAWRF) revealed ameloblastin located along the nonsecretory portion of the Tomes' process of ameloblasts in rat. This localization shows potential interactions with the plasma membrane. Further work needs to be completed in this area to determine if ameloblastin truly has cell binding and/or signaling capabilities.

As one can determine from this background literature review, much remains unknown about the ameloblastin protein. This dissertation adds important scientific evidence to this field. The cloning and sequence of a bovine ameloblastin cDNA resulted in the identification of a new, previously unreported splice variant (Chapter 2). This cDNA sequence also led to bioinformatics analysis, leading to a current hypothesis that the phosphorylation of these splice variants acts as a biochemical switch during tooth formation (Chapter 2). The immunoperturbation experiments (revealed in chapter 3) are the first to supply evidence regarding a functional role of the ameloblastin protein. Further experiments in this field resulted in the establishment of a primary "ameloblast-like" cell culture system (Chapter 4, 5, and 6), and the important role calcium plays in the regulation of these cells (Chapter 5).

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## Chapter 2 Identification of the bovine ameloblastin gene sequence and a new alternative splice variant

### Introduction

The enamel protein ameloblastin has been implicated in amelogenesis imperfecta, a disease affecting the normal formation of enamel [1]. Ameloblastin mRNA has also been found in tissue isolated from an ameloblastoma tumor [2]. The significant lack of data on this protein makes it hard to establish whether ameloblastin is associated with disease, or is simply found in disease associated with aberrant tooth development.

The ameloblastin cDNA sequence is a relatively new sequence in the field of enamel development, with the rat ameloblastin sequence first published by Krebsback and coworkers [3]. Soon after Krebsbacks's initial publication, two groups published similar findings, one in the same species (rat) and one in porcine [4, 5]. These publications have led to three names for this protein, ameloblastin, amelin, and sheathlin [3-5]. Subsequently, three other groups isolated ameloblastin from two different species, mouse and human. In all cloned species the authors have reported two predicted splice variants of this protein.

The full sequence of ameloblastin quickly shed light on protein purification work that had been previously completed. Numerous publications had isolated fragments of ameloblastin, however, never the parent protein [6-9]. Our laboratory had also isolated one of those fragments, and used this information to create an anti-ameloblastin peptide antibody that is used in this report [10].

Little functional data was generated through these previous studies. They did show that ameloblastin is quickly degraded after secretion [11]. The N-terminus of this protein maps to the sheath space[5, 7, 11], and a 27- and 29- kDa fragment bound calcium [8]. This information coupled with sequence data implicating cell binding domains in mouse and rat [4], still left a large void of knowledge in this important field.

This study was designed to sequence the bovine ameloblastin cDNA, and to use this information in bioinformatics analysis to determine conserved regions including cell binding sequences. An antibody to ameloblastin was also used to confirm the presence of the translated protein in developing bovine teeth.

## Materials and Methods

### Identification of bovine ameloblastin cDNA through library screening

*Primer and probe development*: Bovine ameloblastin cDNA was isolated from a bovine enamel organ cDNA library previously described by Li and co-workers [12]. Prior to my sequencing of bovine ameloblastin cDNA, both the porcine and rat sequences had just been published. I used homologous areas in the porcine and rat ameloblastin cDNA sequence to generate oligonucleotide primers. These primers (5' GAAATGGCGGGAGGAAGAGGA labeled SU1 (upstream primer) and 5' GGGGTCATCAGTGGGTCAGCA labeled SL1 (downstream primer)) were used to amplify a portion a 374 base pair sequence from the bovine ameloblastin gene by PCR. PCR primer development was aided with the use of Oligo software (Plymouth, MA). All

PCR reactions were done on a DeltaCycler II thermacycler (Ericomp, SanDiego, USA) under the following conditions: step 1=94°C for 3 minutes; step 2=30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute; step 3=72°C for 7 minutes; step 4= 4°C hold. The 374-bp sequence generated by PCR was then used as a probe for the screening of a bovine non-amelogenin enamel organ cDNA library [12].

The probe amplified by PCR was purified from a low melting point agarose gel (1%) using Promega's Wizard PCR preps DNA purification system (Madison, WI, USA). The probe was then ligated into pCR™ II vector using a TA cloning kit (Invitrogen, Carlsbad, CA, USA). This plasmid with probe insert was then transformed into INV-F' cells (Invitrogen) which were plated on LB agar plates with 1% ampicillin. A colony was picked containing the insert and grown in LB broth with 0.1% ampicillin. DNA from the culture was purified using Promega's Wizard Plus Maxipreps system. The probe was cut from the vector using EcoR1 and purified from a 1% low melting point gel using Promega's Wizard PCR preps DNA purification system.

*Library screening*: XL-1 blue MRF' cells (Uni-ZAP™ XR cloning kit, Stratagene, La Jolla, CA) were prepared as host bacteria (in LB with 0.2% maltose, 10 mM $MgSO_4$ and mixed with a non-amelogenin bovine tooth library at approximately 31,000 plaque forming units [pfu]). The infected bacteria were then mixed with NZY top agar and plated onto NZY plates for library screening. The bovine ameloblastin specific probe was labeled and hybridized using the ECL direct nucleic acid and labeling protocol (Amersham Life Sciences, Piscataway, NJ). Two positive clones (confirmed by PCR)

from the primary screening were picked, regrown and plated on NZY plates for secondary screening. Tertiary screening was performed following the same protocol. Tertiary screening showed a preponderance of positive clones and three clones were isolated and grown at 37 $^{0}$C overnight.

*DNA isolation and purification:* An ExAssist helper phage (Stratagene, La Jolla, CA, USA) was incubated with the XL-1 blue MRF' host cells containing the cloned gene. This protocol allows the *in vivo* excision and recircularization of the vector into a phagemid (pBluescript SK(-) phagemid) containing the cloned insert. This phagemid was then isolated and transformed into SOLR cells (Stratagene). The protocols followed were the Statgene Exassist helper phage protocols, with SOLR strain (Stratagene). The SOLR cells from two clones were grown in LB without a supplement. The inserts were then checked by PCR for the bovine ameloblastin gene using the primers (SU1/SL1) developed for the probe. These primers were also used with the vector primer (SL1 and rM13 / SU1 and T7 primers) to confirm the approximate size and orientation of the insert. DNA purification was then performed following Promega's Wizard Plus Maxipreps system (Promega).

*Sequencing*: Purified DNA was sent to the UCSF sequencing center for sequencing using M13 R and T7 vector primers. Primers were then synthesized from the completed sequence to continue sequencing in a step-wise approach, to determine the full-length cDNA sequence of bovine ameloblastin. Both strands were sequenced to confirm the

sequence and identify sequencing errors. The primers used are described below (table 1).

| Primers used for sequencing: | | | |
|---|---|---|---|
| Positive strand: | | | position |
| 5' | M13R | CAGGAAACAGCTATGAC | (vector) |
| | 325U | CAACAGCCAGGACAGAAA | 325-342 |
| | 567U | GGGCTTCTTGCTAATCCT | 901-918 |
| Negative strand: | | | |
| | T7 | TAATACGACTCACTATAGGG | (vector) |
| | STOPD | TCAGGGCTCTTGGAAA | 1179-1194 |
| | 207D | GGCTATTGGAAACATC | 540-555 |

**Table 1** Sequence Primers. Primers are underlined in sequence, figure 2.

**Identification of additional missing bovine ameloblastin sequence using PCR:**

*Ligation, plasmid purification and sequencing of the missing CDNA sequence*: The bovine ameloblastin cDNA sequence that I identified by library screening (see above) was missing two different stretches of 15 amino acids, that had been identified in alternatively spliced product in other species. In addition, only one polyadenylation site had been found, while three polyadenylation sites had been reported in the literature [2, 3, 5, 13, 14]. I used PCR primers bracketing the missing sequences to amplify PCR products from the bovine cDNA tooth development library [12]. PCR primers were generated using homologous sequences identified in other species. The primers are described below (table 2).

| Upper primers 5'-3' | Lower primers 5'-3' |
|---|---|
| 1) CCCACCTCTCCCATCACAG – H386U | AAATCCTGGAAACATGGCTC – H892L |
| 2) AGGCCAAGAGAACATGAAACT – H333U | CAGTGAGAAAAATTCTTTATTTAAATT –H1963L |

**Table 2** Primers for splice variant and polyadenylation signal analysis.
Primers in group 1 were made to verify the presence of an alternative splice variant of ameloblastin. The upper primer H386U is within the first 15 amino acid deletion. The upper primer in group 2 was made to a region just upstream from the first 15 amino acid deletion, and of to the last of 3 polyadenylation signals found conserved in the 3' non-coding region of other species.

The PCR reactions were run on a Mastercyler gradient (Eppendorf), following temperature optimization of these primers. The following conditions were optimal and were used for PCR amplification: step 1=94°C for 3 minutes; step 2=30 cycles of 94°C for 30 seconds, 55°-60C for 30 seconds (gradient with a specific temperature in each row of wells), 72°C for 1 minute; step 3=72°C for 7 minutes; step 4= 4°C hold. The PCR reaction products were separated by gel electrophoresis using a 2% low melting point agarose gel. The PCR bands were then cut from the gel and purified using a Zymoclean gel DNA recovery kit (Zymo research, Orange, CA, USA). The purified bands were ligated into plasmid vector pCR2.1 TOPO (Invitrogen). This recombinant vector was then was then transformed into TOP10F' *E. coli*, the bacterial cells plated onto LB ampicillin plates, incubated overnight at 37°C. Individual colonies were picked and grown in LB broth with ampicillin. The bacterial broth was grown for approximately 6 hours at 37°C on an orbital shaker at 225 RPM, or overnight at 30°C at 200 RPM. An aliquot of the bacteria solution with 15% glycerin (after reaching an OD of 1.0) was quick frozen in a dry ice ethanol bath for stock bacteria stored at –80°C. The remaining

bacteria solution was then centrifuged at 5000 RPM for 30 minutes, and the pellet used for plasmid purification following the Wizard lamda purification kit protocol (Promega). PCR amplification was performed using the purified plasmid containing the PCR products as a template to verify the presence of the bovine ameloblastin insert. The plasmid was then sent to Elim Biopharmaceuticals (Hayward, CA, USA) for DNA sequencing using T7 as a primer.

**Bioinformatics analysis of ameloblastin:**

*CDNA sequence analysis*: All cDNA homology sequence analyses were done with the aid of MAC DNASIS software by Hitachi (San Francisco, CA). Bovine ameloblastin was compared to all published ameloblastin, amelin and sheathlin sequences retrieved from the NIH gene data bank.

*Protein sequence analysis*: All predicted protein motifs of the bovine ameloblastin protein sequence were analyzed using Prosite. Prosite (www.expasy.ch/prosite/) is a database of protein families and domains. The bovine ameloblastin protein sequence was used to find biologically relevant sequence patterns that would predict possible modifications and/or a known protein family (if any) that the new sequence belongs to. All other known ameloblastin sequences were then scanned against Swissport (protein database) using Prosite. Clustal X (a sequence alignment program, shareware) was then used to align all ameloblastin protein sequences. After alignment the sequence data obtained from Prosite was used to find conserved patterns of interest. Sequence

alignment done by Clustal X is shown in figure 3. Protein homology sequence analysis, and hydrophobicity was performed with the aid of MAC DNASIS software (Hitachi).

**Ameloblastin localization:**

*Antibody purification:* A synthetic peptide (YGAIFPGFGGMRPRL) corresponding to the first 15 amino acids of the N-terminus of a 27- and 29-kDa ameloblastin protein fragment isolated from bovine enamel extracts was used to make a polyclonal rabbit peptide anti-bovine ameloblastin antibody (Ramb) [10]. I purified the antibody from rabbit serum using a Protein A affinity column. Briefly, 50 mls of rabbit anti-bovine ameloblastin peptide serum was centrifuged at 3000 rpm for 30 minutes. A stir bar was added to the supernatant at 4°C, and 15.65 grams of ammonium sulfate (25% cut) was slowly added to the supernatant to allow precipitation. The solution was allowed to stir gently overnight, and then centrifuged at 3000 rpm for 30 minutes. The precipitate was resuspended in 25 mls of 10 mM Tris (pH 8.0), and transferred to dialysis tubing and dialyzed against three changes of 10 mM Tris (pH 8.0) overnight. The Protein A column was first washed with 10 volumes of 100 mM Tris (pH 8.0), followed by 10 volumes of 10 mM Tris (pH 8.0) of loading buffer. The dialyzed Ramb antibody was then loaded onto a protein A affinity column. The Ramb antibody was then eluted from the column by 100 mM glycine (pH 3.0) elution buffer. One mM Tris (pH 8.0) was quickly added to the antibody elution until the purified antibody solution was at neutral pH. A UV-Vis spectrophotometer (wavelength = 280) was then used to establish the antibody concentration using 1 OD = approximately 0.8 mg/ml.

*Immunohistochemistry:* Bovine calf teeth were collected at a local slaughterhouse and kept on ice. The developing teeth (impacted) were dissected from alveolar bone, fixed in 10% non-buffered formalin (NBF) overnight and demineralized in a 5% EDTA solution (changed every 5-7 days). The decalcifying solution was checked by atomic absorption until it showed no traces of [$Ca^{2+}$], confirming complete demineralization. The demineralized teeth were then washed with running $H_2O$ for 1 hour, and then washed with 70% ethanol over an hour with 3 changes of solution. The teeth were then dehydrated through graded ethanol baths followed by xylene and paraffin. The paraffin embedded teeth were sectioned for immunohistochemistry (IHC) analysis.

Five micron sections were cut from the demineralized paraffin embedded teeth and mounted on a glass slide with out a cover slip for immunohistochemical staining. Some sections were stained with either a H and E or trichrome stain for tissue orientation and analysis of mineralization patterns. Ramb (anti-ameloblastin) antibody concentration was optimized by the use of multiple dilutions of antibody.

A Vectastain kit (Vector Laboratories, Burlingame, CA, USA) was used for antibody staining. The tissue sections were blocked in horse serum (15 ul in 1 ml PBS), excess serum removed, and incubated with primary antibody (Ramb) for 2 hours at $37^9$ C. The tissue sections were washed in PBS three times for 5 minutes. Diluted biotinylated secondary antibody (Universal Kit, Vector Laboratories) in PBS was then added to the tissue sections for 2 hours at $37^9$ C.. The cells were then washed in PBS three times for 5 minutes. Vectastain Elite ABC reagent was made and allowed to sit for 30 minutes

(Vectastain Kit, Vector Laboratories), then added to the tissue sections for 30 minutes. The tissue sections were washed in PBS three times for 5 minutes. The cells were stained with a peroxidase substrate solution containing DAB for approximately 5 minutes (Vectastain kit). The tissue section was then mounted with a coverslip and analyzed by light microscopy.

## Results:

### Sequence information on three bovine ameloblastin splice variants, identical except for different patterning of two 15 amino acid inserts.

*Library screening and sequence analysis:* Approximately 85% of all clones isolated from the bovine non-amelogenin cDNA library were positive for ameloblastin. The cDNA sequence isolated from a positive clone was 1443 base pairs, and is predicted to be 397 amino acids. In this report I refer to this sequence as PB397 and shown in figure 2. The first methionine codon found in bovine ameloblastin cDNA is designated as base 1, which is the likely translation start site with the appropriate translation start site consensus sequence [15]. Using the first methionine as the translation start sequence, this bovine ameloblastin encodes an open reading frame of 397 amino acids, with a predicted signal peptide and cleavage site after the first 32 amino acids. The predicted molecular weight of PB397, not including the signal peptide, and without post-translational modifications, is 39.3 kD, with an isolelectric point of 4.97. PB397 is rich in proline (13.6%), leucine (10.3%), and glycine (10.1%).

PCR was used to identify sequence information that was found in other species, but missing from the sequenced bovine clone. This sequence information included two other polyadenylation sites and two other splice variants of bovine ameloblastin (figure 2). Thus, through library screening and PCR analysis a total of three splice variants of bovine ameloblastin were identified (figure1). The full-length cDNA predicting a translation of 427 amino acids with a MW of 42.6 kD without the signal peptide or post-translational modifications was identified. The second splice variant was a 412 amino acids sequence containing a 15 amino acid deletion from amino acid residues 104-118, (PB412), with a predicted MW of 41.3 kDa without the signal peptide or post-translational modifications. Lastly, a predicted 397 amino acid sequence, containing two 15 amino acid deletions from amino acid residues 104-118 and 231-245, that I cloned by library screening (see above) was a novel splice variant, not previously identified in other species. (Figure 1).

**Homology**

Sequence homology between all cloned species revealed a highly conserved ameloblastin cDNA and protein. Bovine ameloblastin showed the highest homology with porcine, followed by human. The highest homology was found in either the 5' or 3' non-coding region followed by the coding cDNA and finally the protein. Full-length bovine ameloblastin (PB427) was used in the homology comparisons with the equivalent full-length found in all other species (Table 3).

| | | Non-coding region | | |
|---|---|---|---|---|
| **Bovine** | **cDNA** | **3'** | **5'** | **protein** |
| **Porcine** | **83%** | **92%** | **87%** | **83%** |
| **Rat** | **70%** | **76%** | **85%** | **66%** |
| **Mouse** | **73%** | **78%** | **82%** | **66%** |
| **Human** | **76%** | **85%** | **80%** | **67%** |

**Table 3.** Bovine sequence homology comparisons using PB428
All comparisons were done using MacDNAsis Software

**Ameloblastin protein sequence alignment and predicted motifs**

*Bovine ameloblastin predicted protein motifs (PB428):*

The bovine ameloblastin protein sequence contains a number of potential myristoylation sites (residues 56-61, 185-190, 278-283, 327-332, 338-343, 357-362, 379-384). Other predicted protein motifs include: two casein kinase II phosphorylation sites (residues 240-243, and 392-395), an RGD cell binding sequence (residue 248-250), and a tyrosine kinase phosphorylation site (97-104). An ameloblastin clustal alignment is shown in figure 3 with the conserved protein motifs.

Sequences encoding predicted phosphorylation sites of ameloblastin were identified and compared by sequence alignment (see diagram of sequence alignment, figure 3). These sites include three possible phosphorylation sites: a tyrosine kinase (Tyr K), a casein kinase II (CKII), and protein kinase C (PKC). Two of the predicted phosphorylation sites

(Tyr K and CKII) have a close relationship with the ameloblastin splice variants (figure 4 and 5).

**Hydrophobicity:**

Hydrophobicity plots [16] were made and compared using full-length bovine ameloblastin (PB427) and splice variant #3 (PB397), containing two 15 amino acid deletions from amino acid # 104-118 and 231-245). The region in the splice variant containing the casein kinase II phosphorylation site (amino acid 231-245) is hydrophobic and predicted to be internalized or membrane bound (figure 6).

**Ameloblastin localization:**

*IHC:*

The antibody to the bovine peptide ameloblastin fragment stained enamel, predentin, and the dentino-enamel junction (DEJ). Immunostaining was strongest at the DEJ and could be detected even at the most dilute concentration (1:500) (figure 7). The enamel exhibited two distinct staining patterns. The inner two-thirds of secretory stage enamel showed positive stained bands, alternating with negative stained bands in a striped pattern. These bands ran perpendicular to the DEJ, and ended two-thirds through the enamel, thus no staining was seen in the outer one-third of enamel adjacent to the ameloblasts (figure 8). This striped pattern was also observed with tri-chrome staining showing alternating blue and red stains within the enamel matrix (figure 9). During later

transition stage enamel formation, staining for the ameloblastin protein was observed at the edge of the mineralized enamel through the full thickness of the enamel (figure 10).

*Western blot:*

Using the anti-ameloblastin peptide antibody (Ramb), Western blot analysis was performed on developing enamel extracts. In the first experiment different concentrations of primary and secondary antibody were used to optimize the reaction revealing a 27 and 29 –kDa protein band positive for the anti-ameloblastin antibody with an alkaline phosphatase labeled secondary antibody. The most intense reaction was revealed with a 1:100 primary antibody dilution (Ramb), followed by a 1:2000 goat anti-rabbit secondary antibody, with weaker reactions seen at greater dilutions of primary and secondary antibody. The negative control with PBS alone followed by staining revealed no bands, and little background. The positive control using a bovine anti-amelogenin antibody revealed an intense reaction showing positive staining of the 20-30 –kDa bands. (figure 11).

A second experiment using a Western blot with increased sensitivity (chemiluminescence) to try and identify higher molecular weight ameloblastin fragments was completed on developing bovine enamel extracts. The chemiluminesence assay showed similar results to the color reaction described above. It was revealed by increased antibody dilutions that this Western assay was more sensitive than the color reaction, however a higher MW ameloblastin protein was not revealed. Although lane 3 (figure 12)

did show signs of a higher MW fragment that was positive for Ramb, it was dismissed as artifact because it did not show up in the lower dilutions (lanes 6,7). (figure 12).

A third Western blot experiment was completed using bovine developing tooth enamel organs rather than the developing enamel as reported in the above experiments. The rationale included the use of the cellular material for protein isolation, before ameloblastin was degraded in the enamel matrix environment by enamel proteases [17-20]. Previous studies have supported higher MW ameloblastin fragments in the enamel organ [21-23].

These studies revealed that a higher MW protein could be detected in bovine enamel organ extracts by using an anti-ameloblastin specific antibody (figure 13). This analysis was done using both the color reaction (alkaline phosphatase labeled secondary antibody), and chemiluminescence (data no shown). The approximate MW of these bands include 130-, 83-, 63-, multiple bands between 30-42, 29-, and 27-kD. Numerous MW bands have shown to react positively when probed with anti-ameloblastin antibodies [21-23]. The most intense reaction occurred at approximately 63 kD. It was also revealed that a more intense reaction with less background was seen when blocking solution (5% nonfat dried milk, 0.1% tween in TBS) was used overnight as opposed to overnight blocking and diluting the antibody in blocking solution (Lane 1 versus 2, figure 13). The Western also revealed that the high MW products recognized by Ramb could only be seen in enamel organ extracts and not enamel extracts, whereas an anti-bovine amelogenin antibody revealed positive bands in both tissue extracts.

## Discussion:

### Isolation of bovine ameloblastin cDNA

I used a bovine cDNA library to screen for the ameloblastin gene, and to amplify cDNA sequences missing from this cloned sequence. This PCR amplification of missing sequences in the ameloblastin clone, confirmed the presence of similar sequences to that found in the full-length and previously identified splice variant of other species. In addition, I used PCR primers to confirm the presence of two other polyadenylation signals in bovine ameloblastin cDNA. These studies have identified a unique ameloblastin splice variant (PB397), which is missing the first 15 amino acids of exon 6 and exon 12. The exon/intron boundaries of ameloblastin were identified through genomic sequencing by Toyosawa and coworkers [2].

This newly identified splice variant is unique in that two highly conserved sequences are spliced out of the full-length ameloblastin cDNA, which is presumably translated into a functional protein. The literature currently supports only two alternatively spliced patterns for ameloblastin, the full-length and a sequence missing the first 15 amino acids of exon 6 [2, 3, 5, 13, 14], but not missing exon 12 as found in bovine. It is not clear if this unique splice variant is bovine specific, or has not been identified in other species. The relative abundance of this splice variant has not been determined, leading to the question of whether this splice variant is expressed or even important in tooth development. Further studies are necessary to determine the abundance of this splice variant, and if this splice variant is conserved across species.

The predicted full-length protein (PB427) contains a number of protein motifs. The conserved sequences, however, are most likely to predict possible functional domains within this protein. Three conserved sequences encoding phosphorylation sites of ameloblastin were identified and compared by sequence alignment. These sequences predicted a tyrosine kinase (Tyr K), a casein kinase II (CKII), and protein kinase C (PKC).

A tyrosine kinase phosphorylation site contains a lysine or arginine amino acid, seven residues to the N-terminal side of the tyrosine to be phosphorylated. An acidic residue (Asp/Glu) is often found at either three or four residues to the N-terminal side of the tyrosine (-Lys/Arg-$(Xaa)_2$-Asp/Glu-$(Xaa)_3$-Tyr-) [24, 25]. Casein kinase II phosphorylation sites are defined as -Ser/Thr-$(Xaa)_2$-Asp/Glu(MacDNAsis software, Hitachi, Ca). Protein kinase C sites are defined as Ser/Thr-Xaa-Arg/Lys (MacDNAsis software, Hitachi, Ca).

The second predicted splice variant of bovine ameloblastin is 412 amino acids and is referred to as PB412 in this report. It matches the full-length protein (PB427) except for a 15 amino acid deletion from the beginning of exon 6. This splice variant has been found in rat, porcine, mouse and human [2, 3, 5, 13, 14]. This 15 amino acid deletion is 100% conserved, with high conservation extending 9 amino acids to the N terminus and 15 amino acids to the C terminus. This amino acid conservation is also interesting in that the splice variant deletes a tyrosine from the spot of a potential tyrosine kinase

phosphorylation site (see figure 5). Dephosphorylation of this site (through deletion) would impart a potentially different function to the splice variant with an aspect of control through phosphorylation. This high conservation in and around this 15 amino acid deletion suggests functional significance. The functional significance of the alternative splice variant controlling the tyrosine phosphorylation site remains intriguing, and further studies are necessary to elucidate a possible mechanism in tooth development.

The third predicted splice variant of bovine ameloblastin is 397 amino acids (PB 397), and contains two 15 amino acid deletions described earlier. The first 15 amino acid deletion is described above, and is identical to that found in PB412. The second deletion has never been reported, and holds the casein kinase II phosphorylation site. This CKII site remains 100% conserved through all cloned species, and includes a high degree of sequence homology in the surrounding amino acids [2, 3, 5, 13, 14]. The CKII phosphorylation site was predicted to be membrane bound or internalized through hydrophobicity charting (figures 3 and 6). Its conservation points to a likely role in the function of this protein, while the hydrophobicity plot may give a glimpse of a possible mechanism. The phosphorylation of ameloblastin could be a key regulator of mineralization, as phosphoproteins have proven to be very important in this mechanism [26]. It also remains feasible that the phosphorylation site remains buried (due to its internal hydrophobic stretch), and upon degradation becomes activated. This phosphorylation site could then be involved in the mineralization mechanism or play a signaling role related to the ameloblasts membrane. A casein kinase II inhibitor has been

reported to perturb tooth formation [27, 28]. Further studies are needed in this area to determine the functional significance of this splice variant.

The full-length and alternative splice variants of the bovine ameloblastin predicted protein sequence all contained an RGD cell binding sequence from amino acid residues 248-250. Some investigators have argued that ameloblastin acts as a signaling molecule based on sequence data [3, 4]. Both rat and mouse ameloblastin contain two cell-binding sequences: DGEA (an integrin recognition sequence) and VTKG (a thrombospondin-like cell adhesion domain) [4]. The porcine and human ameloblastin cDNA contain no known cell-binding sequences. In one study an antipeptide antibody (NKVHOPOVHNAWRF) just downstream from the DGEA cell-binding domain showed that ameloblastin was located along the non-secretory portion of the Tomes' process of ameloblasts in rat and hence shows potential interactions with the plasma membrane [11].

The lack of conservation of the sequences (DGEA, VTKG, and RGD) across species, given the high homology of this protein, may lead one to hypothesize that ameloblastin does not have signaling capabilities. However, unmapped cell-binding sequences in porcine or human may exist. Also, a signaling mechanism involving the ameloblastin protein may not rely on the cell-binding sequences, but be related to the potential phosphorylation of this protein. Further work needs to be completed in this area to determine if ameloblastin truly has cell binding and/or signaling capabilities.

**Nucleotide sequence including highly conserved non-coding region**

The bovine ameloblastin cDNA coding sequence remains highly conserved among all cloned species, ranging from 70% homology in rat to 83% in porcine (see table 3). The homology between the non-coding regions is even higher than the homology between coding region cDNA (see table 3). This non-coding homology is somewhat unusual, and must have an important regulatory role to be conserved through so many species separated by hundreds of millions of years of evolution. MacDougall and coworkers reported, by examining different clones screened from a human library, that a specific poly-adenylation signal did not relate to the transcription of a specific splice variant [14]. Further studies are necessary in this area to confirm the role of the non-coding region on splice variant transcription.

The conservation of the 3' non-coding region in other species was used to generate a primer specific to the last of the 3-polyadenylation signals that generated a PCR product in bovine cDNA (primer H1963L). The primer remained 100% conserved across all known species [2, 3, 5, 13, 14]. Due to the conservation in other species it is likely that the primer remained 100% conserved in bovine. By designing a primer in the last of three known poly-adenylation signals, I was able to produce a PCR product containing all three poly-adenylation sites and complete the 3' non-coding region. Although it is possible that there is more 3' non-coding sequence after the last primer in this region it is likely that this is the end of the 3'non-coding region as was found in all other species [2, 3, 5, 13, 14]. Future studies to amplify the 3' end, such as a 3' RACE experiment are necessary to rule out additional alternate poly-adenylation sites.

**Ameloblastin immunolocalization**

The ameloblastin anti-peptide antibody (Ramb) is specific to a peptide sequence found in bovine teeth. Immunostaining was observed at the dentin-enamel junction (DEJ) in early enamel formation. Other investigators have also shown DEJ staining in both rat and human developing teeth using an antibody generated against a fragment of the rat ameloblastin protein (amino acid 206-379) [3, 14]. However our experiments revealed another localization pattern of this antibody observed in the inner two thirds of bovine enamel with a metachromatic appearance, which is different than any results published thus far. It is interesting that tri-chrome staining also shows a banded staining pattern. The tri-chrome pattern is likely mapping areas with differing degrees of calcification. It is possible that the ameloblastin anti-peptide antibody is only recognizing the protein in calcified areas in enamel, since intense immunostaining is also observed at the start of enamel maturation, where enamel calcification occurs more rapidly. Further immunolocalization studies are necessary to understand this interesting staining pattern. Splice variant specific anti-peptide antibodies are also being synthesized for future work, to relate the ameloblastin splicing pattern to localization data.

Another possibility explaining the staining pattern of ameloblastin in bovine developing teeth could be related to poor penetration of antibodies into enamel matrix from paraffin embedded specimens (personal communication, Dr. Yoshiro Takano). The intense staining at the tip, and the metachromatic pattern of the forming enamel is explained by loose packing of the enamel matrix allowing for the penetration of antibodies at these

regions. It is interesting to note that the metachromatic pattern of ameloblastin staining corresponds to that observed by tri-chrome staining (figure 9). This pattern has been observed before in enamel and is referred to as Hunter Shreger bands, these bands are shown to be related to hypomineralized areas in the enamel [29, 30]. Could ameloblastin lead to hypomineralization? Whether the antibody staining pattern is due to poor penetration of the antibody into paraffin embedded enamel, or ameloblastin is specific in this unique pattern still needs to be determined. Further studies using different processing techniques could be important in deciphering the role ameloblastin plays in tooth formation.

**Western blot analysis of ameloblastin in bovine enamel and tooth organ extracts**

Further work to decipher the specificity of the anti-ameloblastin antibody (Ramb) was completed through Western blot analysis. Western blot analysis confirmed the specificity of this antibody to the lower molecular weight fragments (27 and 29 –kDa) [10], as well as higher molecular weight fragments. This work revealed higher and lower molecular weight fragments found in the enamel organ, where only lower molecular weight fragments were found in the enamel matrix extracts. Enamel organ includes the ameloblasts, stratum intermediam, stellate reticulum, as well as connective tissue cells that give rise to teeth [26]. By isolating proteins from this tissue source the proteins would not yet have reached the extracellular environment, and therefore not be exposed to the extracellular proteases involved in enamel formation. The higher molecular weight fragments of ameloblastin have been shown to be rapidly degraded upon secretion [11, 22], this could explain the ability to only recognize lower MW fragments of ameloblastin

after secretion by the ameloblasts. Without further experiments and controls it is possible that the anti-ameloblastin antibody (Ramb) is recognizing non-specific proteins in the enamel organ. The antibody (Ramb) however, was derived from protein sequence data involving the purification of a 27 and 29 –kDa ameloblastin protein fragment, therefore, without further confirmation the specificity of this protein to ameloblastin to these fragments is likely. Further studies using purified ameloblastin protein, or the peptide to which the antibody was made, as controls are important to confirm that all the MW fragments recognized by the Ramb antibody is ameloblastin specifically.

**Conclusion:**

I have found a unique bovine ameloblastin splice variant. I have also confirmed the presence of the full-length and previously identified splice variants found in other species. This third splice variant may remain unique to bovine, or it simply may not have been found in other species. The fact that this third splice contains a deletion removing the conserved casein kinase II phosphorylation site also suggests a unique function. The higher homology in the non-coding region versus the coding region of ameloblastin is interesting and warrants further investigation. An anti-peptide antibody confirmed the presence of this protein in specific patterns in developing teeth. Further immunolocalization data, along with many other studies are needed to elucidate the intricacies and function of this important protein.

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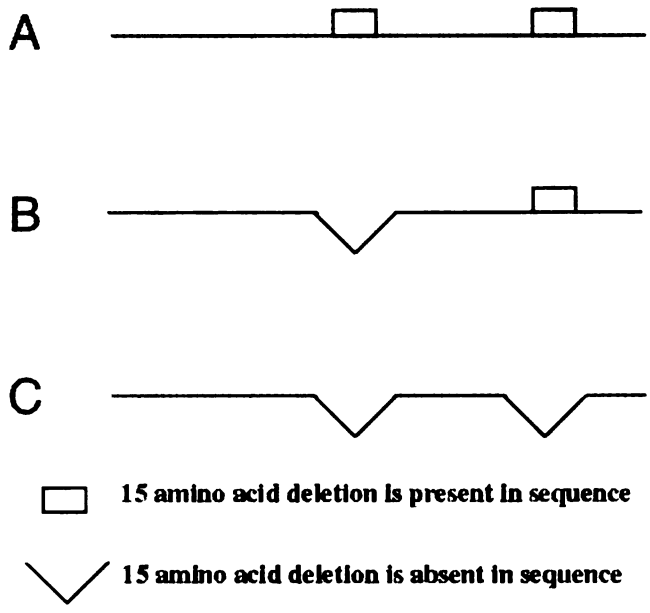


**Figure 1.**
**Bovine ameloblastin splice variants.**
(A) Splice variant #1 contains two 15 amino acid deletions and is predicted to contain 427 amino acids (PB 427). (B) Splice variant #2 contains one 15 amino acid deletion and is predicted to be 412 amino acids (PB412). (C) Splice variant #3 does not contain either of the 15 amino acid deletions and is predicted to be 397 amino acids.

Full sequence of cloned bovine ameloblastin including all three polyadenylation signals:

```
-83                                                    AATTCGGCACGAGCCCAGGCACA
-60  GTCCAGAAAAAATTTTTAGTCTTCTTTTCTTAGAACTATCTTGGTTGGCATCGCAAGGCA

1   ATGAGAGCACAGTGCATGCCAGCATTGAAGATCCCACTTTTCAAAATGAAGGACATGATA
     M  R  A  Q  C  M  P  A  L  K  I  P  L  F  K  M  K  D  M  I  20
61  CTGATCCTGTGCCTCCTGAAAATGAGTTCTGCAGTGCCGGCGTTTCCTCAGCAACCTGGG
     L  I  L  C  L  L  K  M  S  S  A  V  P  A  F  P  Q  Q  P  G  40
121 ATACCAGGCATGGCTAGTTTGAGCCTTGAGACAATGAGACAGTTGGGAAGCCTGCAGGGA
     I  P  G  M  A  S  L  S  L  E  T  M  R  Q  L  G  S  L  Q  G  60
181 TTAAACCTGCTTTCTCAGTATTCAAGATTTGGCTTTGGGAAATCATTTAATTCTTTGTGG
     L  N  L  L  S  Q  Y  S  R  F  G  F  G  K  S  F  N  S  L  W  80
241 ATGAATGGTCTTCTCCCACCACATTCCTCCTTCCCATGGATGAGACCAAGGGAACATGAA
     M  N  G  L  L  P  P  H  S  S  F  P  W  M  R  P  R  E  H  E  100
301 ACTCAACAGTATGAATATTCTTTGCCTGTGCATCCCCCACCTCTCCCATCGCAGCCATCC
     T  Q  Q  Y  E  Y  S  L  P  V  H  P  P  P  L  P  S  Q  P  S  120
361 CTGCAGCCTCAACAGCCAGGACAGAAACCTTTCCTCCAGCCCACCGTTGTCACCTCCATG
     L  Q  P  Q  Q  P  G  Q  K  P  F  L  Q  P  T  V  V  T  S  M  140
421 CAGAACGCAGTCCAGAAGGGCGTACCTCAGCCTCCGATTTACCAGGGACATCCGCCCTTG
     Q  N  A  V  Q  K  G  V  P  Q  P  P  I  Y  Q  G  H  P  P  L  160
481 CAGCAAGCAGAGGGGCCGATGGTTGAACAGCAGGTGGCGCCATCAGAAAAGCCACCAACG
     Q  Q  A  E  G  P  M  V  E  Q  Q  V  A  P  S  E  K  P  P  T  180
541 ACCGAGCTACCAGGAATGGATTTTGCTGATCTACAAGACCCACCGATGTTTCCAATAGCC
     T  E  L  P  G  M  D  F  A  D  L  Q  D  P  P  M  F  P  I  A  200
601 CATTTGATATCTCGGGGACCAATGCCACAAAATAAACCATCTCAGCTTTACCCAGGAATA
     H  L  I  S  R  G  P  M  P  Q  N  K  P  S  Q  L  Y  P  G  I  220
661 TTTTACGTGACCTATGGAGCAAATCAACTGACCGCTCCTGCCAGACTTGGCATCATGAGC
     F  Y  V  T  Y  G  A  N  Q  L  N  A  P  A  R  L  G  I  M  S  240
721 TCAGAAGAAATGGGGGGAGGAAGAGGAGACCCCCTGGCCTATGGAGCCATATTCCCAGGA
     S  E  E  M  G  G  G  R  G  D  P  L  A  Y  G  A  I  F  P  G  260

781 TTTGGAGGCATGAGGCCCAGGCTTGGAGGGATGCCCCACAACCCAGACATGGGTGGGGAC
     F  G  G  M  R  P  R  L  G  G  M  P  H  N  P  D  M  G  G  D  280
841 TTTACTCTGGAATTTGACTCCCCAGTCGCTGCAACCAAAGGCCCGGAGAAGGGAGAAGGA
     F  T  L  E  F  D  S  P  V  A  A  T  K  G  P  E  K  G  E  G  300
901 GGTGCACAAGACTCCCCTGTGCCAGAGGCCCACCTAGCCGATCCGGAAAGCCCAGCTCTC
     G  A  Q  D  S  P  V  P  E  A  H  L  A  D  P  E  S  P  A  L  320
961 CTTTCAGAGCTAGCACCTGGTGCCCTAGAAGGGCTTCTTGCTAATCCTGAGGGCAATATT
     L  S  E  L  A  P  G  A  L  E  G  L  L  A  N  P  E  G  N  I  340
1021 CCCAACCTGGCAAGGGGCCCTGCAGGGCGCAGCAGGGGATTCCTTAGGGGAGTCACCCCA
     P  N  L  A  R  G  P  A  G  R  S  R  G  F  L  R  G  V  T  P  360
1081 GCAGCTGCCGACCCACTGATGACCCCTGGATTAGCTGAGGTTTATGAGACCTACGGTGCT
     A  A  A  D  P  L  M  T  P  G  L  A  E  V  Y  E  T  Y  G  A  380
1141 GATGAGACCACAACTCTGGGTCTCCAGGAAGAAACGACTGTGGACTCCACAGCGACCCCT
     D  E  T  T  T  L  G  L  Q  E  E  T  T  V  D  S  T  A  T  P  400
1201 GACACTCAGCACACATTGATGCCAAGAAACAAGGCCCAGCAGCCCCAAATCAAGCATGAT
     D  T  Q  H  T  L  M  P  R  N  K  A  Q  Q  P  Q  I  K  H  D  420
1261 GCATGGCATTTCCAAGAGCCCTGA
     A  W  H  F  Q  E  P  *                                        427

1285 GAGTCTGGAGATATCGGCTACTTTCTGTATGCACAAGCTCCCCAGCTTTGTCCCCATAGC
1345 ATATCTGTTTGCAAAAACACTTATTACCCTTCTGCAGCAAAGGCATTAAAAGTGCTAAGC
1405 ACATATTAATAAATACAAGTGGCTAGAAATAGTGTAGGTCCCCTTCTTGCTTTCATTCTC
1465 TTATTGAAATAAAATGTGCCGCCTGTCTCTGTGATTTAGAAATACTATTAATAACATCAG
1525 AGCAAGTCAAAGGGTCTCTGCATTTGAAAATCACTGTTTCTTAGCTATCTTGGCATTACA
1585 GAATTTCTCCCGCTAGCATGACACTGTTATATCCAGGAAATGTGACACTACTTTGGAAAT
```

```
1645 TTTTCTTTAAGCAAAGGCACATATTCTTAGAATTATAAGTTATTTCATTCAAACGTTATT
1705 AAATGGGGACTGGTCAACAATCCCTGACTGGTATTACTGGGTTTGGTTTACTGGATTTAA
1765 AATTCTCATTTGTAGAGTATTTTATTTAATCTAGTAAAAACATCTAGCCTAATTTAAATA
1825 AAGAATTTTTCTCACTG
```

**Figure 2.**

**Bovine ameloblastin sequence (PB427).**

The 5' noncoding regions is found from nucleotide –83 to -1. The coding region is found from nucleotide 1 to 1284. The 3' noncoding region is found from nucleotide 1285 to 1841. Sequencing primers are underlined, along with the 3 polyadenylation signals found in this sequence. The vertical line shortly after the first polyadenylation signal in the 3' noncoding region depicts the start of the poly-A tail sequenced from the clone containing the new splice variant. The amino acid sequence underlined contains the peptides used to make the Ramb antibody. The two 15 amino acid sequences missing from the sequenced clone were identified using PCR, and are in bold.

The three bovine ameloblastin splice variants include the full-length shown above containing 427 amino acids, a second splice variant with a 15 amino acid deletion from amino acid 104-118, and a third previously unidentified splice variant missing two 15 amino acid sequences from 104-118 and 231-245. All other species show the full-length and splice variant #2.

*Ameloblastin clustal alignment (PB427):*

```
                     Signal sequence                                    PKC site
bovine          MRAQCMPALKIPLFKMKDMILILCLLKMSSAVPAFPQQPG-----IPGMASLSLETMRQL
porcine         -----MPALKIPLFKMKDMVLILCLLKMSSAVPAFPRQPG-----TPGVASLSLETMRQL
human           -----MSASKIPLFKMKDLILILCLLEMSFAVPFFPQQSG-----TPGMASLSLETMRQL
mouse           -----MSASKIPLFKMKGLILFLSLVKMSLAVPAFPQQPGAQGMAPPGMASLSLETMRQL
rat             -----MSASKIPLFKMKGLLLFLSLVKMSLAVPAFPQQPGAQGMAPPGMASLSLETMRQL
                     *.* ********.::*:*.*::** *** **:*.*      **:************
                                                    Tyr k site
bovine          GSLQGLNLLSQYSRFGFGKSFNSLWMNGLLPPHSSFPWMRPREHETQQYEYSLPVHPPPL
porcine         GSLQGLNMLSQYSRFGFGKSFNSLWMHGLLPPHSSFQWMRPREHETQQYEYSLPVHPPPL
human           GSLQRLNTLSQYSRYGFGKSFNSLWMHGLLPPHSSLPWMRPREHETQQYEYSLPVHPPPL
mouse           GSLQGLNALSQYSRLGFGKALNSLWLHGLLPPHNSFPWIGPREHETQQYEYSLPVHPPPL
rat             GSLQGLNALSQYSRLGFGKALNSLWLHGLLPPHNSFPWIGPREHETQQYEYSLPVHPPPL
                **** ** ****** ****::****::******.*: *: ********************

bovine          PSQPSLQPQQPGQKPFLQPTVVTSMQNAVQKGVPQPPIYQGHPPLQQAEGPMVEQ-QVAP
porcine         PSQPSLQPQQPGQKPFLQPTVVTSIQNPVQKGVPQPPIYQGHPPLQQVEGPMVQQ-QVAP
human           PSQPSLKPQQPGLKPFLQSAAATTNQATALKEALQPPIHLGHLPLQEGELPLVQQ-QVAP
mouse           PSQPSLQPHQPGLKPFLQPTAATGVQVTPQKPGPQPPMHPGQLPLQEGELIAPDEPQVAP
rat             PSQPSLQPHQPGLKPFLQPTAATGVQVTPQKPGPHPPMHPGQLPLQEGELIAPDEPQVAP
                ******:*:*** *****.:..*  * .  *   :**:: *: ***: *    :: ****

bovine          SEKPPTTELPGMDFADLQDP-------------------------PMFPIAHLISRGPM
porcine         SEKPPEAELPGLDFADPQDP-------------------------SMFPIARLISQGPV
human           SDKPPKPELPGVDFADPQGPSLPGMDFPDPQGPSLPGLDFADPQGSTIFQIARLISHGPM
mouse           SENPPTPEVPIMDFADPQFP-------------------------TVFQIARSISRGPM
rat             SENPPTPEVPIMDFADPQFP-------------------------TVFQIAHSLSRGPM
                *::** .*:* :**** * *                          .:* **: :*:**:
                                        CK II site
bovine          PQNKPSQLYPGIFYVTYGANQLNAPARLGIMSSEEMGGGRGDPLAYGAIFPGFGGMRPRL
porcine         PQDKPSPLYPGMFYMSYGANQLNSPARLGILSSEEMAGGRGGPLAYGAMFPGFGGMRPNL
human           PQNKQSPLYPGMLYVPFGANQLNAPARLGIMSSEEVAGGREDPMAYGAMFPGFGGMRPGF
mouse           AHNKASAFYPGMFYMSYGANQLNAPARIGFMSSEEMPGERGSPMAYGTLFPRFGGFRQTL
rat             AHNKVPTFYPGMFYMSYGANQLNAPARIGFMSSEEMPGERGSPMAYGTLFPGYGGFRQTL
                .::* . :***::*:.:******:***:*::****  * * .*:***::** :**:*  :

                     myristyl site
bovine          GGMPHNPDMGGDFTLEFDSPVAATKGPEKGEGGAQDSPVPEAHLADPESPALLSELAPGA
porcine         GGMPPNSAKGGDFTLEFDSPAAGTKGPEKGEGGAEGSPVAEANTADPESPALFSEVASGV
human           EGMPHNPAMGGDFTLEFDSPVAATKGPENEEGGAQGSPMPEANPDNLENPAFLTELEPAP
mouse           RRLNQNSPKGGDFTVEVDSPVSVTKGPEKGEG-PEGSPLQEANPGKRENPALLSQMAPGA
rat             RGLNQNSPKGGDFTVEVDSPVSVTKGPEKGEG-PEGSPLQEASPDKGENPALLSQMAPGA
                  :  *.  *****:*.***.: *****: ** .:.**: **   . *.**:::::: ..

                                                          myristyl site
bovine          LEGLLANPEGNIPNLARGPAGRSRGFLRGVTPAAADPLMTPGLAEVYETYGADETTTLGL
porcine         LGGLLANPKGKIPNLARGPAGRSRGPP-GVTPADADPLMTPGLADAYETYGADETTTLGL
human           HAGLLALPKDDIPGLPRSPSGKMKGLP-SVTPAAADPLMTPELADVYRTYDADMTTSVDF
mouse           HAGLLAFPNDHIPSMARGPAGQR---LLGVTPAAADPLITPELAEVYETYGADVTTPLG-
rat             HAGLLAFPNDHIPNMARGPAGQR---LLGVTPAAADPLITPELAEVYETYGADVTTPLG-
                  **** *:..**.:.*.*:*:      .**** ****:** **:.*.**.** **.:.

bovine          QEETTVDSTATPDTQHTLMPRNKAQQPQIKHDAWHFQEP
porcine         QEEMTMDSTATPYSEHTSMPGNKAQQPQIKRDAWRFQEP
human           QEEATMDTTMAPNSLQTSMPGNKAQEPEMMHDAWHFQEP
mouse           DGEATMDITMSPDTQQPLLPGNKVHQPQV-HNAWRFQEP
rat             DGEATMDITMSPDTQQPPMPGNKVHQPQV-HNAWRFQEP
                : * *:* * :* : :. :* **.::*:: ::**:****
```

**Figure 3.**

**Ameloblastin sequence alignment using Clustal X (sequence alignment tool), with the full-length ameloblastin bovine predicted sequence (PB427) compared to all other full-length cloned species.** The human clone contains a unique repeat not found in other species [1]. Conserved predicted phosphorylation sites are shown. Cell binding sequences are also shown.

* conserved amino acids

: conserved similarities

predicted sequence data

secretory signal sequence

phosphorylation sites

cell binding sequences

Ramb and Camb peptide for antibody

myristyl site

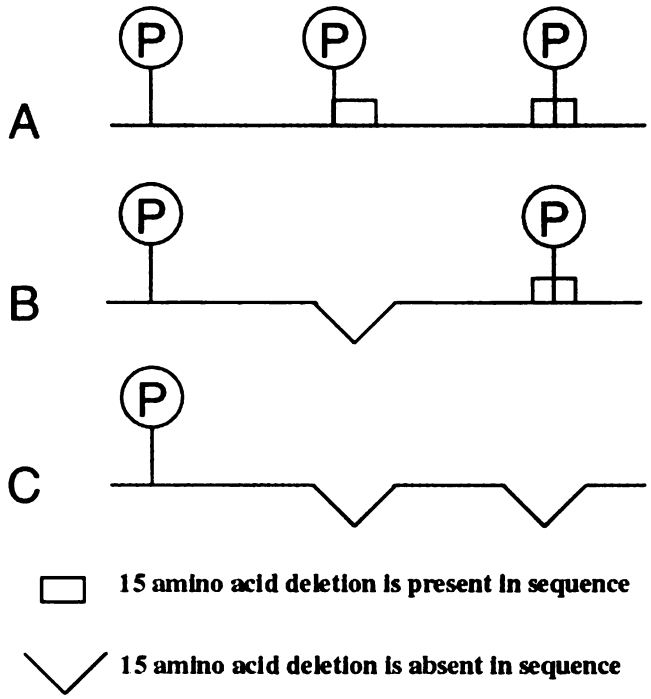


**Figure 4.**
**Diagram depicting predicted phosphorylation sites, and its relationship to the splice variants of bovine ameloblastin.**

(A) Splice variant #1 - Full sequence of bovine ameloblastin containing three conserved phosphorylation sites including two 15 amino acid deletions (PB427). (B) Splice variant #2 - Bovine ameloblastin sequence contains a 15 amino acid deletion with the tyrosine of a tyrosine kinase phosphorylation site (PB412). (C) Splice variant #3 - The bovine ameloblastin sequence that was library screened contains two 15 amino acid deletions, each deletion holding a conserved phosphorylation site (PB397).

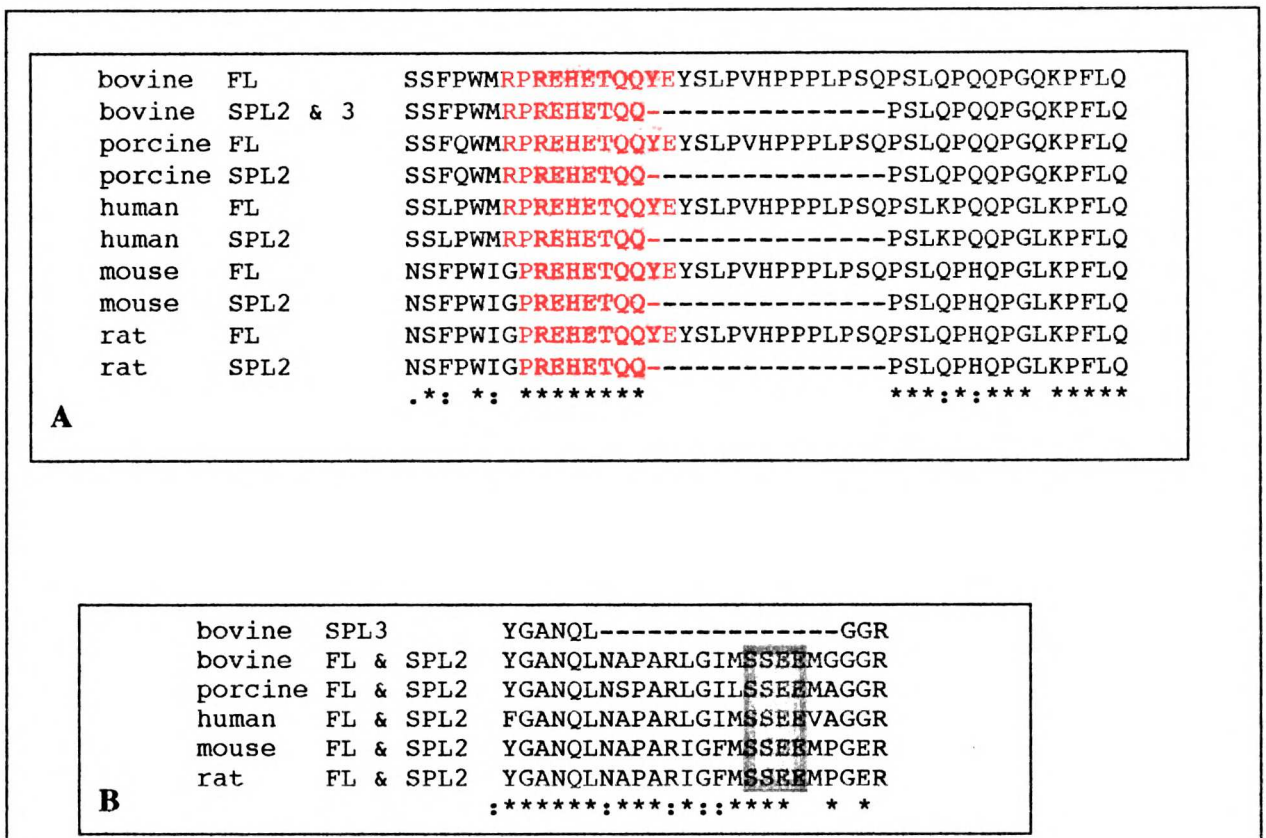


**Figure 5.**
**Clustal alignment of the 15 amino acid deletions leading to different spice variants.**
This sequence information shows amino acid deletions 104-118 (A), and amino acid deletion 231-245 (B).Both deletions can be observed in the predicted full-length protein (PB427) leading to other splice variants. (A) The first 15 amino acid deletion is missing in splice variant #2 (PB412)and splice variant #3 (PB397). (B) The second 15 amino acid deletion is present in the full-length(PB427), and in splice variant #2 (PB412), but missing in splice variant #3.

* conserved amino acids
: conserved similarities
-REHETQQY (tyrosine kinase phosphorylation site sequence, notice the tyrosine (Y) is part of deletion #1). The casein kinase II phosphorylation site is conserved in all species.

FL - full length protein (PB427)
SPL2 - splice variant #2 (PB412)
SPL3 - splice variant #3 (PB397)

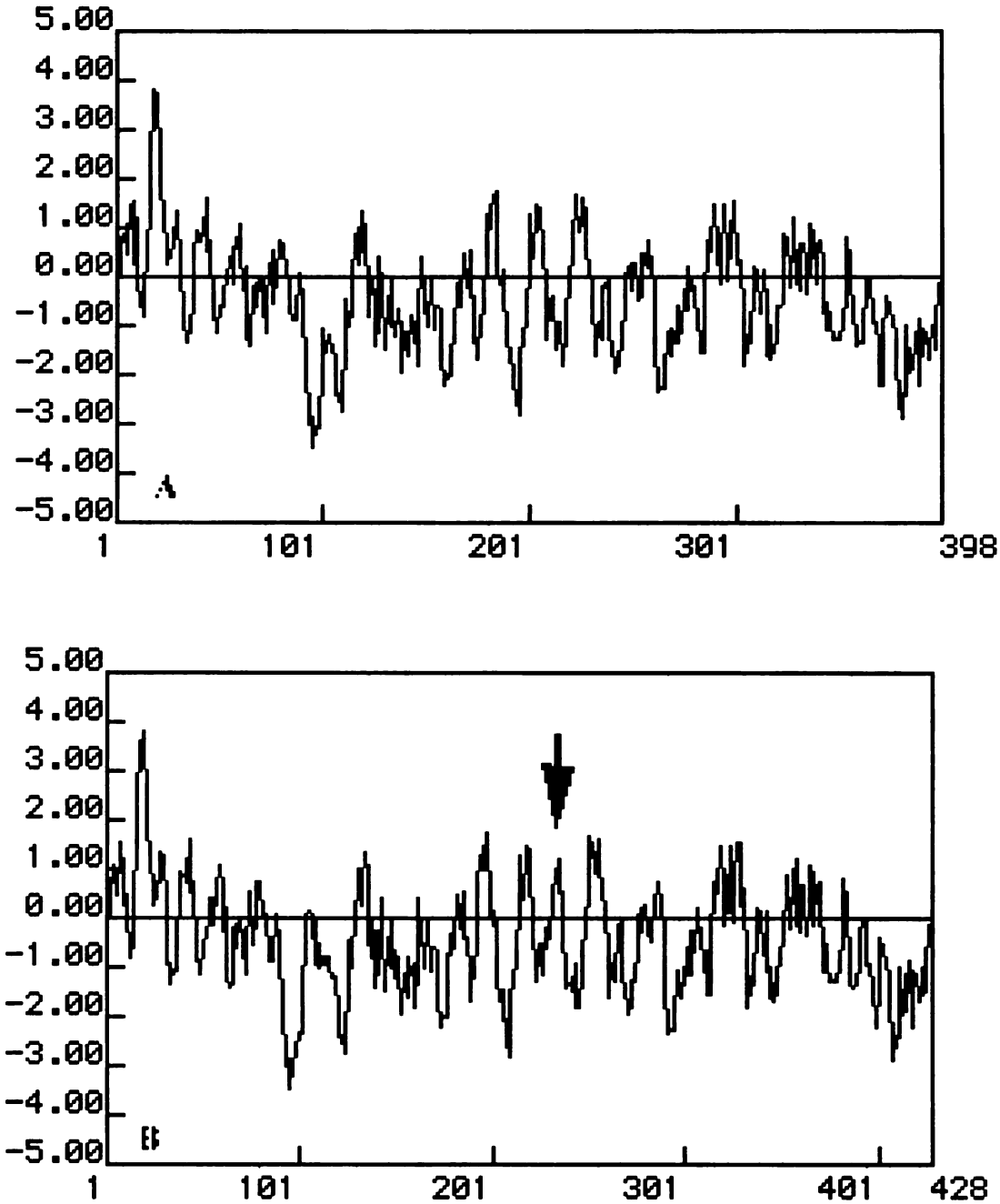


**Figure 6.**
**Kyte and Doolittle hydrophobicity plots of bovine ameloblastin.**
(A) Bovine ameloblastin splice variant #3 (PB397). (B) Bovine ameloblastin splice variant #1 (PB427). The arrow points to the 15 amino acid deletion containing the predicted casein kinase II (CKII) phosphorylation site. This CKII site is predicted to be internalized, or membrane bound by this hydrophobicity plot.

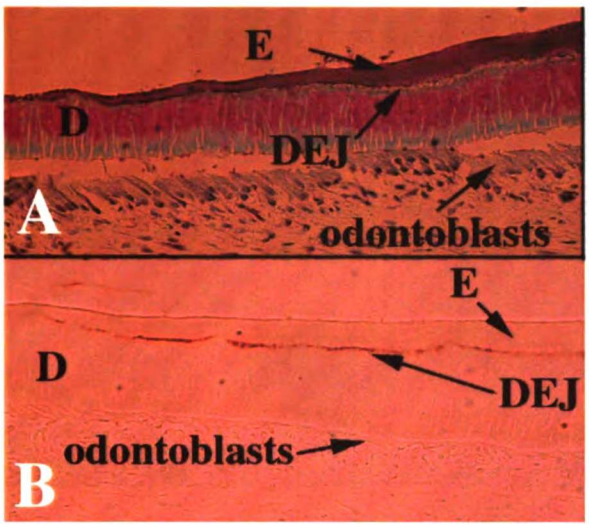


**Figure 7.**
**IHC and trichrome stain of demineralized bovine tooth at early enamel formation.**
(A) Trichrome stain of bovine tooth. (B) Notice specific staining of DEJ with 1 to 100 Ramb (rabbit anti-bovine ameloblastin peptide antibody).

Enamel (E), dentin (D), dentinoenamel junction (DEJ), and odontoblasts are labeled.

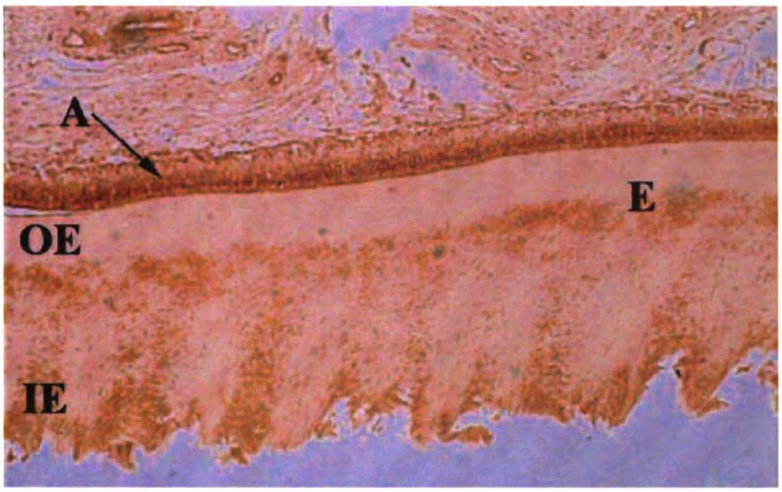


**Figure 8.**
**IHC of demineralized developing bovine tooth stained with 1:25 Ramb (10X).**
Ameloblasts (A), enamel (E), outer enamel (OE), and inner enamel (IE). Ameloblasts show positive staining. The inner two-thirds of enamel also shows positive staining in an alternating pattern. The outer enamel was negative.

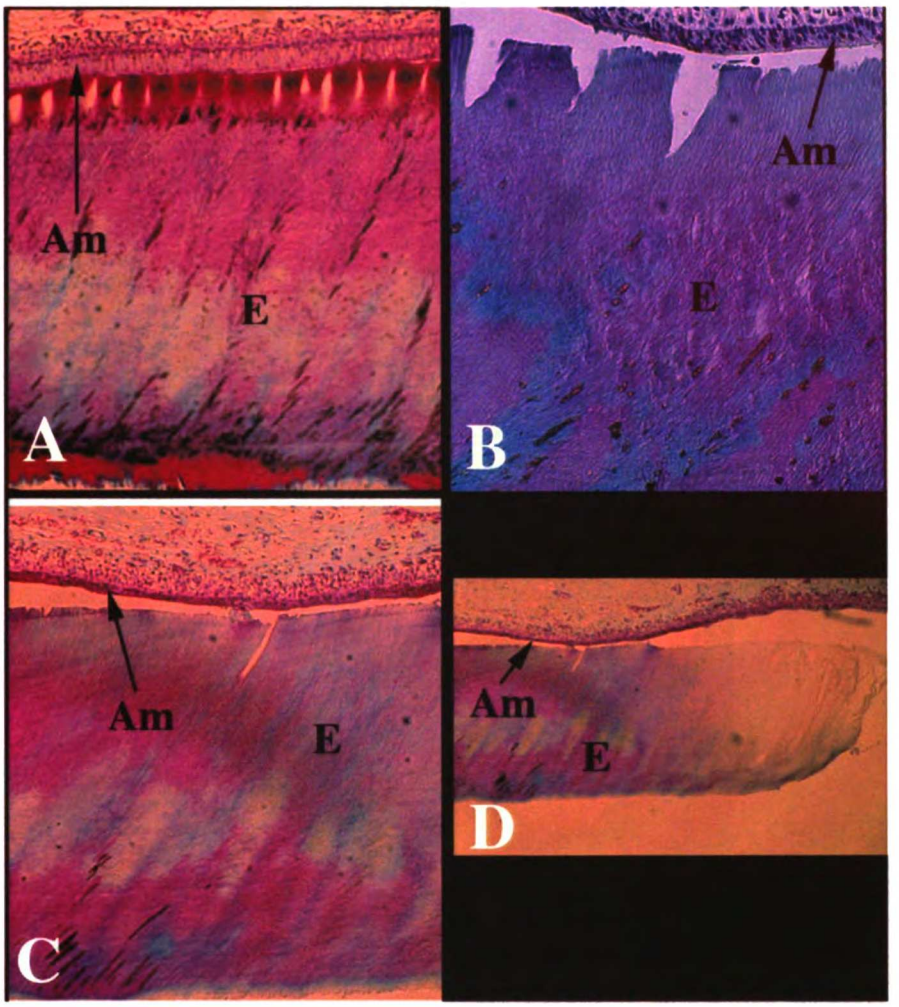


**Figure 9.**
**Trichrome stain of demineralized bovine enamel 20X (A,B), 10X (C), 4X (D).**
Am (ameloblasts). E (enamel). Notice alternating blue and red staining pattern

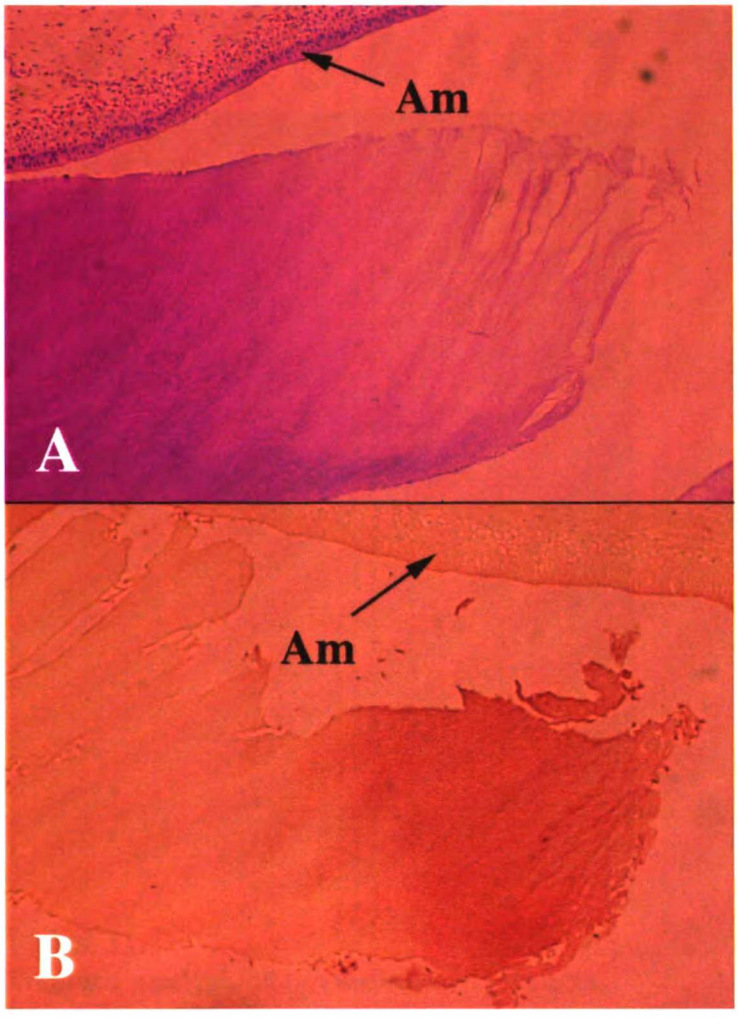


**Figure 10.**
**IHC of developing bovine tooth (10X) at the transition stage.**
(A) H and E stain of the developing tooth. (B) Tooth stained with 1:75 Ramb. (Am) ameloblasts at the transition stage.

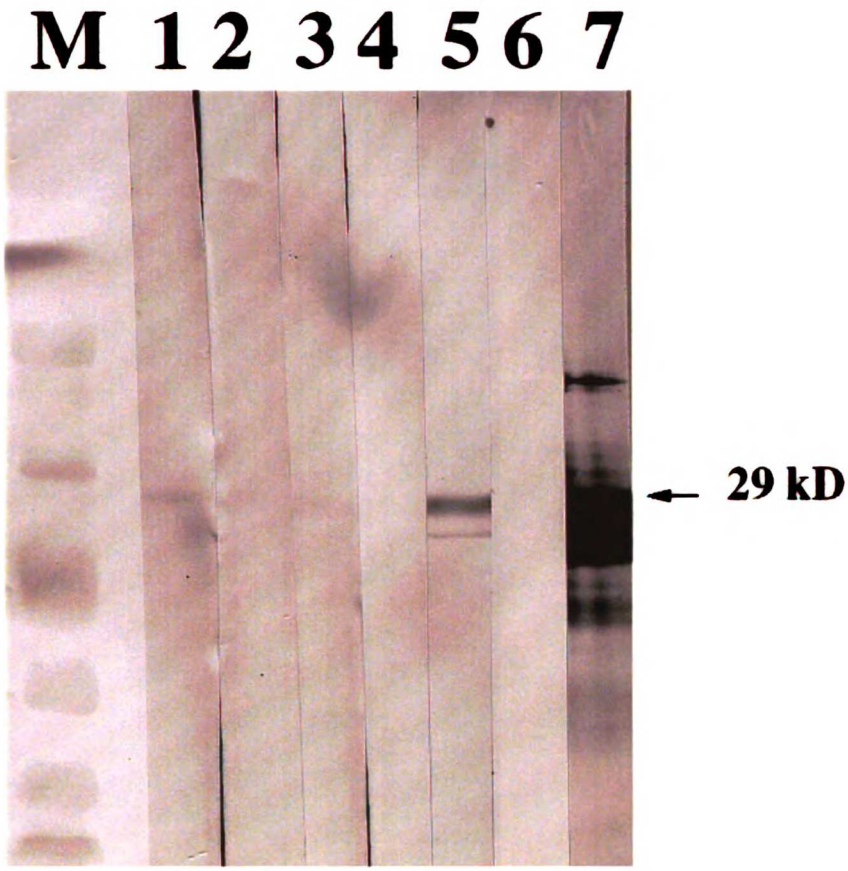


**Figure 11.**
**Western analysis of bovine enamel extracts**
Low MW marker (Lane M).

| | primary Ab | secondary Ab |
|---|---|---|
| Lane 1= | 1:500 Ramb | 1:2000 |
| Lane 2= | 1:500 Ramb | 1:5000 |
| Lane 3= | 1:1000 Ramb | 1:2000 |
| Lane 4= | 1:1000 Ramb | 1:5000 |
| Lane 5= | 1:100 Ramb | 1:2000 |
| Lane 6= | Buffer only control | |
| Lane 7= | 1:2000 Ramg | 1:5000 |

The 27 and 29 –kDa ameloblastin bands were more intense with increasing concentrations of Ramb antibody. The buffer only negative control showed no specific staining. An anti-amelogenin antibody (Ramg) was used as a positive control.

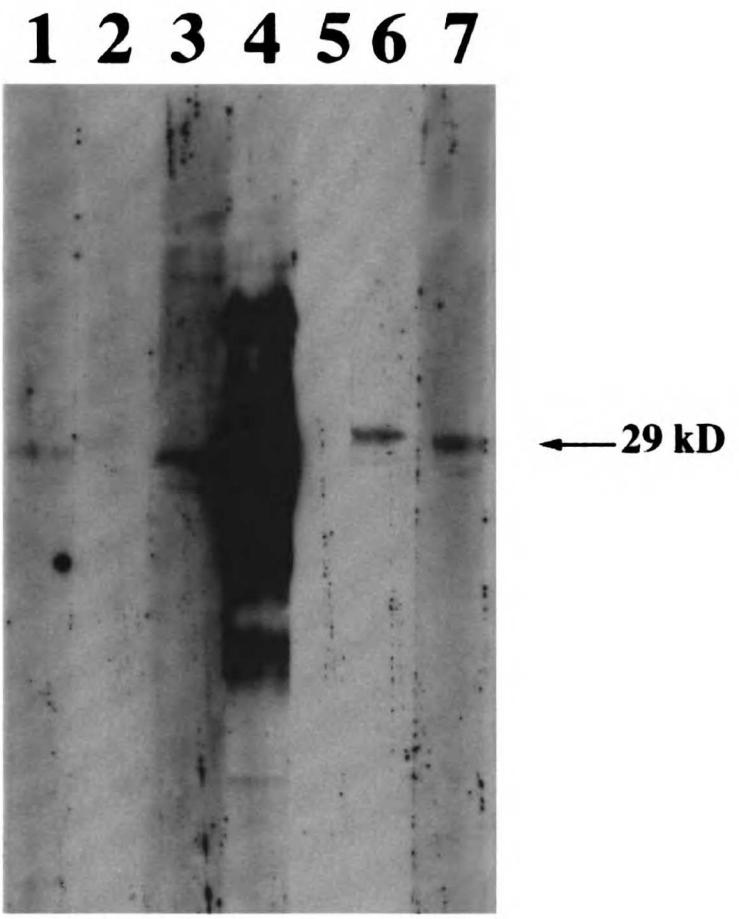


**Figure 12.**
**Western analysis of enamel extracts using chemiluminescence.**

| | primary Ab | secondary Ab |
|---|---|---|
| Lane 1= | 1:2000 Ramb | 1:5000 |
| Lane 2= | 1:3000 Ramb | 1:5000 |
| Lane 3= | 1:2000 Ramb | 1:2000 |
| Lane 4= | 1:1000 Ramg | 1:1000 |
| Lane 5= | buffer only control | |
| Lane 6= | 1:500 Ramb | 1:500 |
| Lane 7= | 1:500 Ramb | 1:2000 |

The chemiluminescence assay proved to be more sensitive than the color assay shown in figure 11, however higher MW bands could not be observed with the Ramb antibody. Lane 3 did show a higher MW band, however this was dismissed as artifact because it did not show up at stronger concentrations of Ramb antibody (Lane 6 and Land 7). The negative control of buffer only showed no specific staining (Lane 5). The positive control using bovine anti-amelogenin antibody (Ramg) showed intense staining for amelogenin (Lane 4).

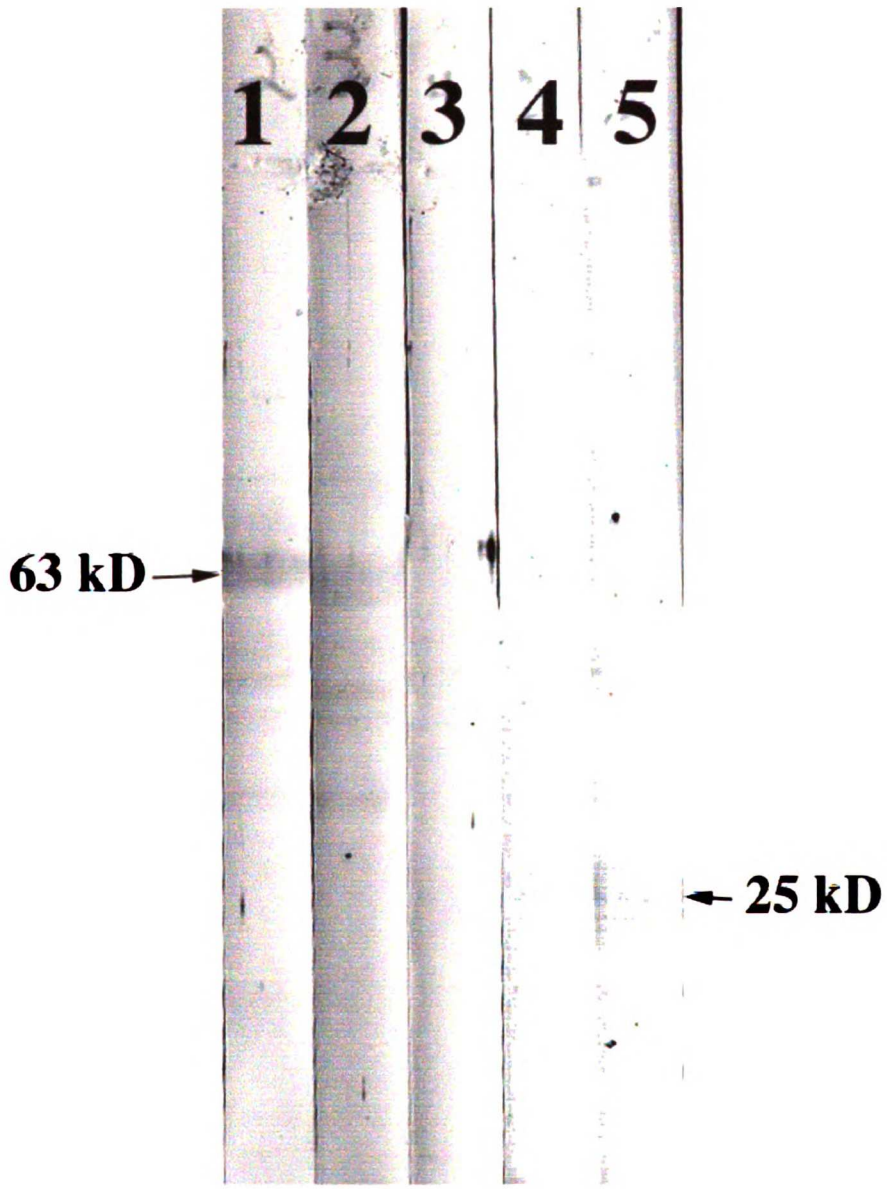


**Figure 13.**
**Western analysis of bovine enamel organ and developing enamel extracts**

Lanes 1, 2, and 4 are enamel organ extracts. Lanes 3 and 5 are extracts from developing enamel. Antibody dilutions include 1:250 Ramb (lanes1-3) and 1:2000 anti-amelogenin controls (lanes 4-5). 1:2000 anti-rabbit secondary antibody was used in all reactions. Lane 2 is the same as lane 1 except the primary and secondary antibodies were diluted in blocking solution instead of TBS.

Higher molecular weight ameloblastin fragments and multiple bands could be observed when enamel organ extracts were used instead of developing bovine enamel extracts.

## Chapter 3. Immunoperturbation of ameloblastin leads to accelerated tooth germ organ culture development.

### Introduction

Ameloblastin is the second most common protein so far identified in the enamel matrix, yet there is only speculation and no significant data to support an actual function of this protein. Ameloblastin cDNA was sequentially cloned by three different groups, leading to three names for the same protein: ameloblastin, amelin, and sheathlin [1-3]. There have been in total five species where ameloblastin cDNA has been cloned and sequenced: rat, porcine, mouse, bovine, and human. Ameloblastin is highly conserved between all species [1-6] (Chapter 2). Ameloblastin cDNA is transcribed into as many as three different splice variants (Chapter 2). I refer to this protein as ameloblastin in this chapter because this name was designated by the group to first publish the full-length clone [3].

Prior to cloning the ameloblastin cDNA, degradation fragments of this protein were studied by immunohistochemical localization. The more stable N-terminus products were localized to the sheath space, hence the name "sheath protein" and sheathlin [7]. The C-terminus of this protein appears to be broken down rapidly and has been shown to primarily localize intensely just beneath the Tomes' process of the ameloblasts becoming more diffuse until disappearing in the outer 50 microns of enamel [8] [2, 9-11].

Ameloblastin mRNA is found from early presecretory to late maturation stage in ameloblasts, and presecretory odontoblasts [12]. Although a 62 and a 70 kD protein

fragment have been identified by Western analysis in the enamel epithelium, only smaller products are found in the enamel matrix [12]. Studies of these degradation products have suggested possible functions of ameloblastin, but have generated more questions than answers.

Fukae and co-workers established that the porcine ameloblastin 27 and 29 kDa fragments bind calcium, but not hydroxyapatite. These authors proposed a role involving the release of calcium leading to initial calcification of the enamel matrix, based on the temporal pattern of these proteins [13]. A recent study of rat ameloblastin extracellular processing has shown that the parent protein, and most of its degradation products are soluble in simulated enamel fluid [14]. This same study revealed that while some of the degradation products (17, 16, 15, and 14 kD) are thought to form insoluble aggregates, only small amounts of the remaining 17 and 16-kDa fragments appeared to be bound to enamel crystals [14].

First, since most ameloblastin fragments are not likely crystal bound their involvement in enamel crystal formation is diminished [14]. Second, ameloblastin mRNA is found in both preodontoblast and differentiating to late maturation stage ameloblasts in a temporally distinct manner, indicative of a possible involvement in cellular differentiation [15]. Lastly, the parent protein and C-terminus degradation products of ameloblastin are found adjacent to the ameloblasts, in a region suggesting cellular control [8]. I propose that ameloblastin has a significant role in ameloblast signaling for the above reasons.

In this study I analyzed the role of ameloblastin in ameloblast differentiation through the use of a tooth organ culture system. Immunoperturbation using an antibody against a conserved peptide sequence of ameloblastin was used to detect how the disruption of this ameloblastin protein, in enamel formation, would affect ameloblast differentiation.

## MATERIALS AND METHODS

### Enamel organ culture:

Pregnant mice were sacrificed by cervical dislocation, and the developing tooth germs were micro-dissected from the mandibles of the mouse embryos at day E16.5 (embryonic day 0 starting at the first sight of vaginal plug). The dissections were done under aseptic conditions in a petri dish containing Hanks' solution (Nissui, Japan) in which Penicillin (100units/ml), Streptomycin (mg/ml), and Fungizone (0.25 mg/ml). Whole first mandibular molar tooth germs were cultured using the modified Trowel organ culture system [16]. The tooth germs were placed on sterile Millipore filter paper over stainless steel mesh grids in 18 mm plastic disposable culture dishes. Four tooth organs were cultured per dish, and tooth organs were removed for processing at different time intervals. These time intervals included day 4, 6, 8, 10, 12, 14, 15, 16, and 18. These intervals allowed me to follow the development of enamel throughout its developmental stages.

The tooth germs were suspended at the air-medium interface. The dishes were incubated at 37°C in 5% $CO_2$ and 21.5% $O_2$ in BGJb medium (Sigma) supplemented with 1%

buffer (2 mM glycine, 2 mM glutamine, 0.5 mM L-ascorbic acid), and 1% antibiotics (100 units/ml penicillin, 100 µg/µg streptomycin, 0.25 µg/ml fungizone). Medium was changed every 2 days. The E16.5 tooth germs were cultured for a total of 18 days. The tooth germs were then fixed and stored in 4% paraformaldehyde at $4^0$C until further processing for histological analysis (see table 1 for days and number of samples, below). E16.5 tooth organs were grown with 10% FCS (fetal calf serum) and without FCS supplemented media. The tooth organs were also grown with and without anti-ameloblastin antibody (0.1% Ramb antibody, 2.84 µg/ml).

| | FCS(+), AB (-) | FCS(+), AB (+) | FCS(-), AB (-) | FCS(-), AB (+) |
|---|---|---|---|---|
| at day 4 | 0 | 2 | 5 | 2 |
| at day 6 | 0 | 2 | 2 | 2 |
| at day 8 | 3 | 2 | 6 | 2 |
| at day 10 | 0 | 0 | 3 | 0 |
| at day 12 | 3 | 2 | 5 | 2 |
| at day 14 | 0 | 2 | 3 | 2 |
| at day 15 | 3 | 0 | 2 | 0 |
| at day 16 | 0 | 0 | 4 | 0 |
| at day 18 | 6 | 2 | 0 | 2 |
| at day 20 | 3 | 0 | 0 | 0 |
| total tooth germs | 18 | 12 | 24 | 12 |

**Table 1**
**Sample size of cultured tooth germs.**
The tooth germs were cultured and fixed for analysis at different days. The tooth germs were cultured with or without serum (FCS) and with or without antibody (Ab). The table above shown the number of tooth germs that were cultured and showed either a "normal" developmental response when cultured without antibody, or an accelerated developmental response when cultured with Ramb antibody. The teeth germs were cultured during five different experiments and the numbers represent total numbers of tooth germs showing results from all five experiments.

Cultured tooth germs were further processed for histological analysis. Cultured tooth germs treated with and without the antibody were pre-fixed with 1/2 Karnovsky fixative solution (2.5% glutaraldehyde and 2% paraformaldehyde) in 0.1M phosphate buffer (pH

7.4), rinsed with 0.1M phosphate buffer (pH 7.4) containing 6.16% sucrose. The specimens were post-fixed with 1% osmium tetroxide in 0.1M phosphate buffer (pH 7.4) at 4°C for two hours. After dehydration with a graded series of ethanol, the specimens were embedded without decalcification in Epon 812 resin. Semi thin sections (1 μm) were stained with toluidine blue for observation under the light microscope.

**Statistical analysis:**

The cultured tooth germs were analyzed by histological examination. As reported in the results an accelerated rate of tooth development was noted when the teeth were grown in experimental media. A positive was assigned to the tooth if they showed an accelerated rate of tooth development, and a negative if they did not show an accelerated rate of development. The accelerated rate of tooth development was defined by histological markers. The histological markers are reported in the results and are defined as initiation of enamel matrix formation, stages of ameloblasts, and formation and mineralization of the enamel matrix. The tooth germs were analyzed to determine if the difference in accelerated rate of development versus "normal" controls during culture were statistically different. The experimental tooth germs were compared to the control tooth germs only if they were sampled at the same day (i.e. day 8 experimental germs were compared to day 8 control tooth germs). This data was then used with a Mantel-Haenszel Chi-square test for statistical analysis to establish statistics over the entire time course of the experiment.

**Histological analysis:**

The antibody used in organ culture and immunohistochemistry was a rabbit anti-bovine ameloblastin polyclonal antibody (Ramb). The antigen (YGAIFPGFGGMRPRL) for these antisera was a synthetic peptide corresponding to the first 15 amino acids of the N-terminus of a 27- and 29-kDa ameloblastin protein fragment isolated from bovine enamel extracts. [17]. The antibody was purified from rabbit serum using a Protein A affinity column.

*Immunohistochemsitry of teeth grown in vivo:*

Mice were sacrificed by cervical dislocation, the mandible dissected, and divided in half. The tissue was rapidly frozen in liquid nitrogen, and oblique sections cut from the freshly frozen and processed mandible yielding serial sections of mouse incisor. Indirect immunostaining (ABC method) was performed on sections after brief ethanol fixation, and counterstained with methyl green. A polyclonal anti-ameloblastin antibody (1:50 dilution) was used in immunostaining.

**RESULTS:**

**Immunohistochemistry**

Immunostaining of mouse incisor cross sections, using the Ramb antibody, revealed an intense immunoreaction that was localized exclusively over the enamel layer at the stage

of matrix formation (figure 1). Control sections of the mouse incisor processed similarly, but incubated without primary antibody revealed no immunostaining (figure 1).

**Tooth germ organ culture:**

*Organ culture of day E16.5 mice in serum medium:*

Control experiment

The control E16.5 tooth germs (figure 2) revealed a typical timeline of enamel maturation in a tooth germ organ culture. Predentin formation occurred by day 4 of culture. Calcification of the dentin matrix, and the start of enamel matrix formation was noted by day 8 of culture. Secretory ameloblasts were well developed by day 10, and differentiated into maturation stage ameloblasts by day 14. Full enamel thickness overlain by late secretory stage ameloblasts was seen by day 12 (figure 3). Day 18 showed maturation stage ameloblasts with a thick highly mineralized enamel layer (figure 4).

Neutralizing experiment (with antibody and 10% FCS in culture medium)

Histological analysis in the neutralizing experiments showed that the initial formation of the enamel matrix occurred earlier, beginning at day 6, rather than at day 8 as found in the control experiments (figure 2). At day 12 the ameloblasts were at the transition stage in the neutralizing experiments, unlike the control cultures, which were still in the secretory and late secretory stage at this time (figure 3). The transitional stage ameloblasts occur just before maturation stage ameloblasts and histologically shows a reduction in height and a shift of the organelles to the distal part of the cell, where

secretory stage ameloblasts show Tomes' process [18]. Maturation stage ameloblasts were also found at the occlusal end of the developing tooth cusp by day 12 in the neutralizing experiments. In contrast, maturation stage ameloblasts were not seen until day 14 in the control experiments. The neutralizing experiments resulted in a mature enamel that exhibited signs of normal mineralization by day 18 (figure 4). The phenotype generated by the neutralizing antibody in the E16.5 tooth germs seemed to show an advanced stage of development by approximately two days. However, the phenotype was more subtle than the tooth germs grown in serum free media. The anti-ameloblastin antibody in the organ culture medium seemed to lead to an accelerated differentiation and development of ameloblasts in the tooth germs.

*Organ culture of day E16.5 mice in serum-free medium:*

Control experiment (organ culture in serum-free medium)

Culturing tooth organs in serum free media shows a considerable delay in tooth development, relative to tooth germs cultured in a serum containing media [16, 19, 20]. In the control tooth germs I cultured in serum free media, dentin mineralization and enamel formation did not always occur, and the enamel layer was much thinner than in the organ culture of tooth germs in a media with serum (E16.5 with FCS). Presecretory ameloblasts and unmineralized dentin (predentin) were still present by day 8 with no enamel formation (figure 5), in contrast to the control tooth germs cultured with serum where dentin mineralization was observed by day 8. The serum free culture conditions did produce some mineralized dentin that could be observed by day 12 and day 14 with a

thin enamel layer (figure 6 and 7). Although enamel did not always form, a thick unmineralized enamel layer was observed by day 16 in one specimen (figure 8).

Neutralizing experiment (serum free culture medium supplemented with antibody)

In the serum free culture of the E16.5 tooth germs, the addition of anti-ameloblastin antibody (Ramb) to the culture media accelerated development of ameloblasts and formation of dental hard tissues relative to the controls. Dentin mineralization and enamel matrix formation was initially observed in these serum free (Ramb+) tooth germs at day 8 and continued throughout the remainder of the culture (figure 5 and 6). Even though enamel development was generally delayed in serum free conditions. In the serum free control experiments, dentin mineralization was not observed by day 8. At day 8 the tooth germs cultured in serum free conditions with Ramb more closely resembled the developmental stage of E16.5 control tooth germs grown with serum at day 8. These same experimental tooth germs produced a thick enamel matrix by day 14 (two days prior than the serum free controls, figure 7), and formed a highly mineralized enamel layer by day 18 of organ culture (figure 8).

Statistical analysis:

The tooth germs grown in media containing Ramb showed an accelerated stage of development that was statistically significant by a Mantel-Haenszel Chi-square test, compared to the controls showing "normal" development of tooth germs in culture with a p value > .001. Each day that was analyzed showed different types of accelerated

development depending on the developmental stage of the tooth germs. This accelerated tooth development is reported above in the results.

**Discussion:**

The results of this study show that an antibody against ameloblastin, when used in the media of tooth organ cultures, led to an advanced stage of tooth development by approximately 2 days in both serum containing and serum free media. This advancement of tooth development occurred in the presence of anti-ameloblastin peptide antibody (Ramb) made to a 15 amino acid peptide, derived from the bovine ameloblastin sequence [17]. The usefulness of this antibody in the mouse organ culture was confirmed by immunohistochemistry of mouse incisor development in vivo.

Sequence alignment reveals 7 of the 15 bovine amino acids to be identical to mouse, and 5 of the remaining 8 to hold conserved similarities (Table 2). This conservation proved to generate a recognizable epitope by the polyclonal antibody between bovine and mouse species. This result was shown through immunohistochemistry of mouse incisors.

```
bovine YGAIFPGFGGMRPRL
mouse  YGTLFPRFGGFRQTL
       **::** :**:*   :
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* conserved amino acids
: conserved similarities

**Table 2.**
**Sequence alignment of bovine amd mouse amino acids containing bovine peptides that were used to make the Ramb antibody .**

The use of a tooth organ culture system has been a valuable tool in the study of epithelial-mesenchymal interactions during mandibular morphogenesis. These studies have

primarily used anti-sense molecules showing that inhibition of growth factors led to aberrant development of the tooth organ [21-23]. However, Mitsiadis and coworkers successfully used immonoperturbation with an antibody to the differentiating factor midkine in the tooth organ culture system to inhibit morphogenesis and cell differentiation [24]. The successful use of antibody in immunoperturbation of organ culture may involve the "leaky" ameloblasts as a method of antibody delivery to the enamel layer.

The enamel organ has been shown to allow the diffusion of molecules through the cells to the newly formed enamel [18]. Much of this work has been done with smaller molecules such as calcium [25], however larger molecules (i.e. Hrp peroxidase 44,000 Mw) have also been shown to penetrate the enamel layer after only 5 minutes following intravenous injection [26, 27]. Studies showing the penetration of relatively large molecules has only been published in studies of maturation stage smooth ended ameloblasts, though it has also been observed in secretory stage ameloblasts (personal communication, Yoshiro Takano). The diffusion of these larger molecules provides a possible route of penetration with the anti-ameloblastin specific antibody used in this analysis. Although a previous study has used an antibody for immunoperturbation of tooth development [24], future studies are necessary to determine the validity of the delivery of the ameloblastin antibody to the target cells and enamel matrix.

The response of ameloblasts to serum-free versus serum containing cultures was markedly different. Although the use of serum free cultures has been reported [28, 29],

other investigators have shown that serum is essential in the development and mineralization of cultured tooth germs [16, 19, 20, 30]. In this study the development of the tooth germ advanced by two days in the presence of media containing serum and Ramb (anti-ameloblastin antibody). This advanced development showed initial enamel formation at 6 days, whereas control tooth organs (without Ramb) did not show enamel formation until 8 days. The advanced stage of tooth development continued throughout all cultures containing Ramb.

An advanced stage of enamel formation in the antibody containing media, relative to tooth germs without antibody, was also seen in the tooth germs cultured in serum free media. To determine the relative effects that growth factors present in serum would have on cultured tooth germs, the tooth germs were also cultured in serum free media (with and without Ramb). The advanced stage of tooth formation had a stronger phenotype in the serum free conditions with Ramb versus serum containing media. It is possible that when serum growth factors are present, they help overcome some of the effects that occur when ameloblastin is bound to an antibody.

The first reports published in the literature regarding tooth germ culture conditions reported serum as an essential component for tooth development and mineralization [19, 20]. Contrary to these earlier studies a serum free tooth germ culture has been established [28, 29]. Although earlier claims showed lack of enamel formation in tooth germs cultured in serum free media, newer reports have demonstrated enamel matrix formation (without mineralization) in up to 97% of their cultures in a chemically defined

serum free conditions [20]. The results obtained in our experiments with serum free conditions do not correlate with the high success rates reported. My results show only thin enamel formation even by day 14, and sometimes a thicker enamel matrix formation that does not mineralize in control tooth germs grown in a serum free media. These results correlate well with others in the lab (Dr. Terashima, personal communication), as well as with earlier literature [16, 19, 20]. The tooth germs cultured with antibody (Ramb+) in the media, on the other hand were dramatically different. Enamel formation was observed by day 8, thick enamel formation by day 14, and mineralized enamel by day 18, even in serum free conditions. In this experiment when ameloblastin was blocked by immunoperturbation with the Ramb antibody in culture, ameloblasts showed accelerated differentiation and increased enamel formation.

The mechanism behind the accelerated tooth development, when ameloblastin protein was blocked in organ culture, is unknown since factors responsible for ameloblast differentiation in teeth have not yet been identified. However, sequence data for the mouse shows that ameloblastin may have a direct protein to cell receptor type signaling event [1]. Both rat and mouse ameloblastin contain two cell binding sequences: DGEA (an integrin recognition sequence) and VTKG (a thromabospondin like cell adhesion domain) [1]. Although bovine ameloblastin contains one known cell binding sequence (RGD) [unpublished data, chapter 2 of dissertation], both porcine and human ameloblastin do not. The cell binding sequences or a yet unknown protein binding domain remains a possible mechanism by which ameloblastin is controlling ameloblast

differentiation. Further work needs to be completed in this area to determine if ameloblastin truly has cell binding and/or signaling capabilities.

Another possible function of ameloblastin in ameloblast differentiation may be related to its calcium binding properties [13]. Extracellular calcium has been shown to control the differentiation of epithelial cells such as keratinocytes, which are highly regulated by external calcium. Levels of 0.05-0.12 mM calcium are necessary for normal growth, whereas, concentrations above 1.2 mM lead to terminal differentiation [31].

Calcium levels close to keratinocytes are present in developing enamel near the ameloblasts[32]. Aoba and Moreno have found that the total calcium concentration in secretory enamel matrix averages about 0.50 mM, with 0.053 mM in the enamel fluid [32]. They have used the same data to calculate the amount of free calcium in enamel as 0.153 mM. The concentration of calcium localized to the ameloblasts is not known, however, Aoba and Moreno established a range very similar to the range that controls keratinocyte differentiation *in vitro*. Further studies are necessary to examine the possible role ameloblastin has in local calcium concentration controlling ameloblast differentiation.

The results of these experiments show that ameloblastin affects enamel growth and ameloblast differentiation. Binding of ameloblastin by the anti-peptide antibody seems to block downstream suppression events that regulate the differentiation of ameloblasts. Blocking ameloblastin allows enamel to reach maturation much faster than the control

organ cultures. Ameloblastin, however, may not be a negative, but a positive regulator of ameloblast differentiation. It is possible that the blocking of the antibody keeps ameloblastin from further degradation, locking non-degraded ameloblastin in a positive regulatory role. Further studies are necessary to decipher the negative suppression or positive stimulatory role of ameloblastin. Further controls in these type of experiments, such as the use of ameloblastin specific peptides, or ameloblastin protein are essential to prove that the effects of this experiment are directly related to the specificity of the antibody to ameloblastin. These types of controls will help prove that the accelerated development is not due to cross-reaction of the antibody, or to the increase of protein in the media leading, but to the effects of ameloblastin antibody binding.

**Conclusion:**

Blocking of ameloblastin protein (or degradation peptide) by an antibody against a sequence highly homologous to mouse ameloblasts caused the accelerated differentiation of ameloblasts leading to an increased enamel matrix thickness in organ culture. In serum containing media (E16.5 (FCS+, AB+)) this phenotype was subtle, however, these same experiments revealed a more dramatic phenotype in serum free culture conditions. I hypothesized that ameloblastin immunoperturbation in an organ culture system would elucidate a role of ameloblastin on tooth development. The results of this study show an acceleration of ameloblast differentiation in the absence of ameloblastin, supporting my hypothesis. In conclusion, the enamel protein ameloblastin is linked to the differentiation and accelerated formation of the enamel matrix, however, further studies are necessary to confirm and decipher the function(s) of ameloblastin.

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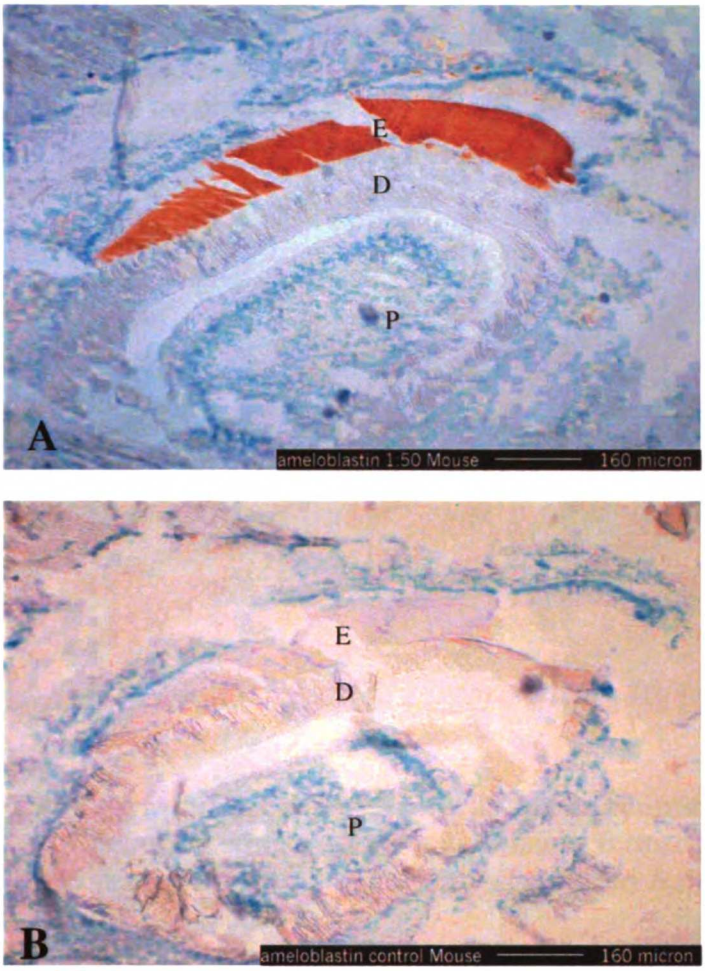


**Figure 1**
**IHC on frozen mouse incisor sections with anti-ameloblastin antibody.**
(A) Ramb antibody (1:50 dilution) shows an intense immunoreaction in the mouse incisor enamel. (B) Control section is done without primary antibody (PBS) and reveals no immunostaining.

Enamel (E), dentin (D), and pulp (P).

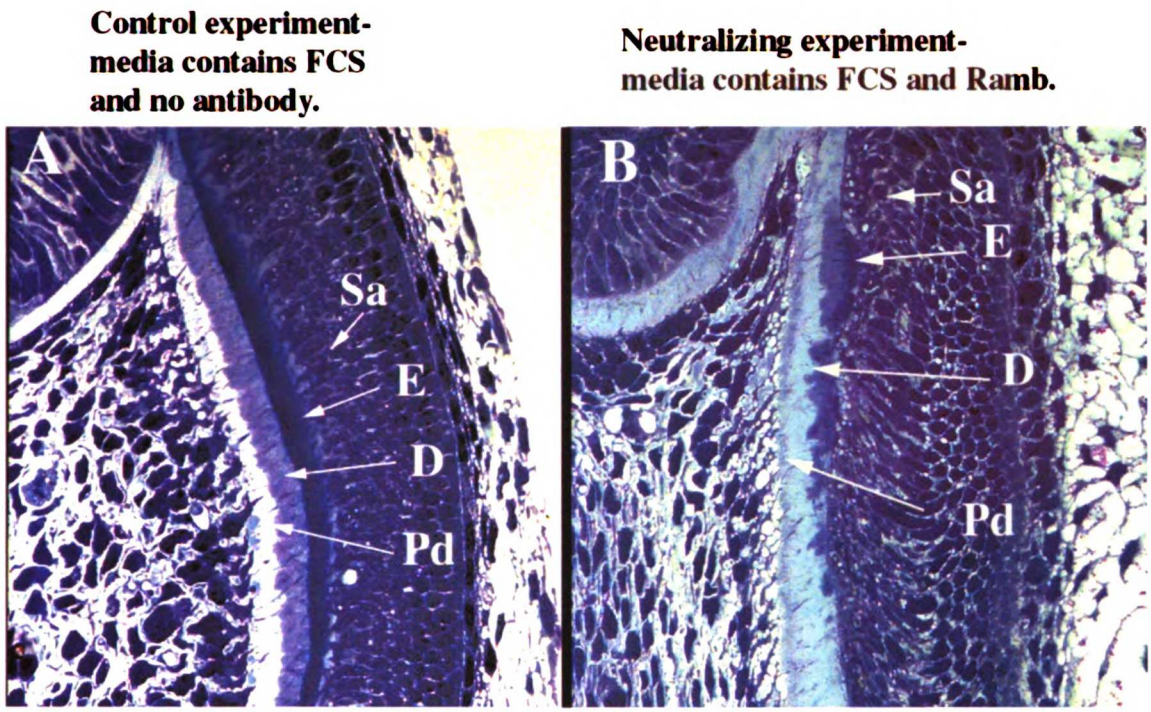


**Figure 2.**
**Tooth germ culture experiment with media containing serum.**
(A) Control experiment shows initial enamel formation at day 8 . (B) Neutralizing experiment shows initial enamel formation at day 6.

Enamel (E), Dentin (D), Predentin (Pd), Secretory stage ameloblasts (Sa).

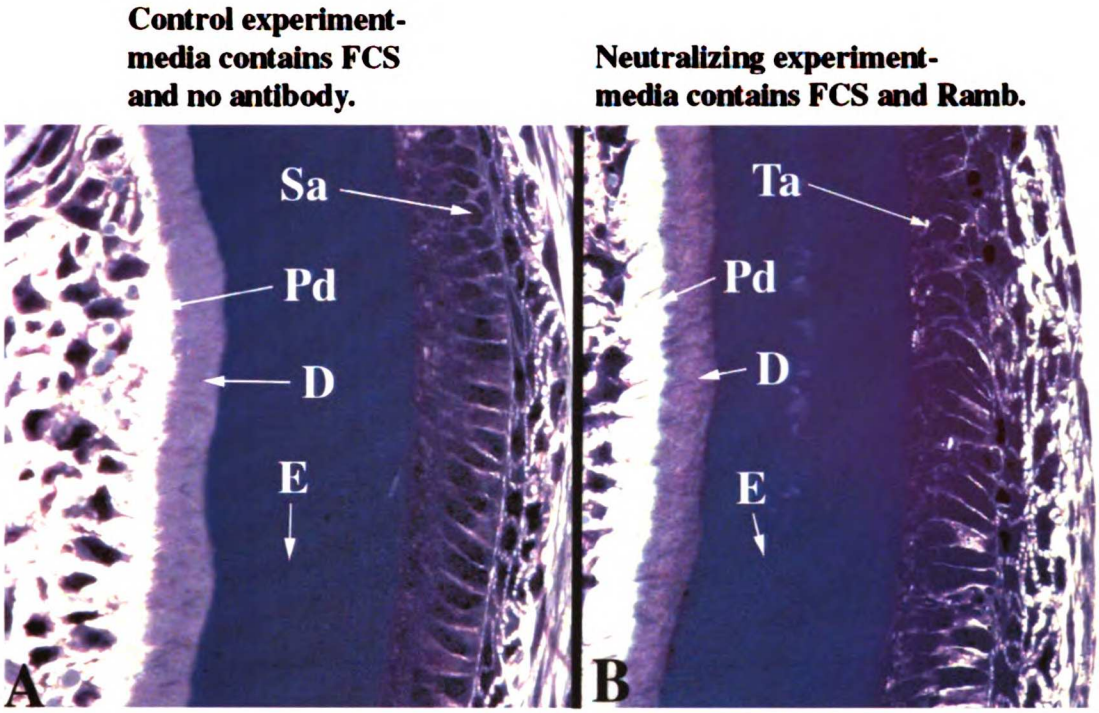


**Figure 3.**
**Tooth germ culture experiment with media containing serum.**
(A) Control experiment at day 12 shows an thickened enamel matrix with ameloblasts at the secretory stage. (B) Neutralizing experiment at day 12 shows a thickened enamel matrix with ameloblasts at a later stage (transition) than those shown in A. Transition stage ameloblasts lose the Tomes' processes, and become shorter, with the organelles shifting to the distal part of the cell.

Enamel (E), Dentin (D), Predentin (Pd), Secretory stage ameloblasts (Sa), Transition stage ameloblasts (Ta).

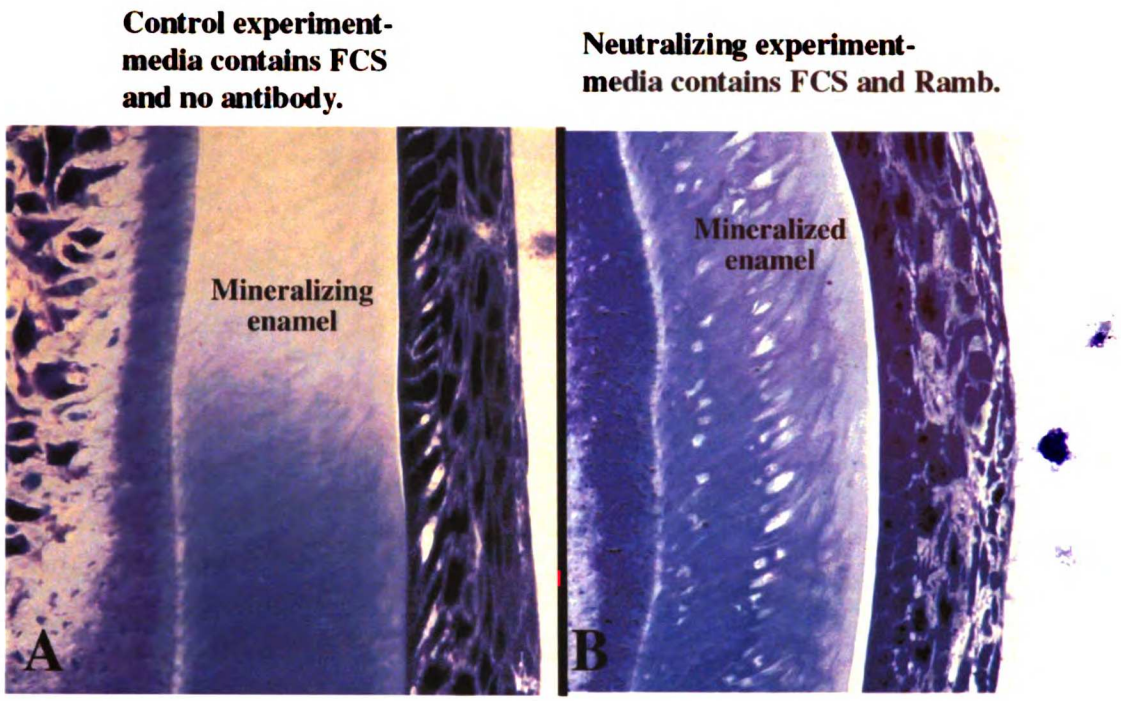


**Figure 4.**
**Tooth germ culture experiment with media containing serum.**
(A) Control experiment at day 18 shows a full thickness of the enamel matrix with enamel still mineralizing. (B) Neutralizing experiment at day 18 shows a full thickness of the enamel matrix with enamel more mineralized than that shown in A. The more mineralized enamel is shown with the lighter blue stain.

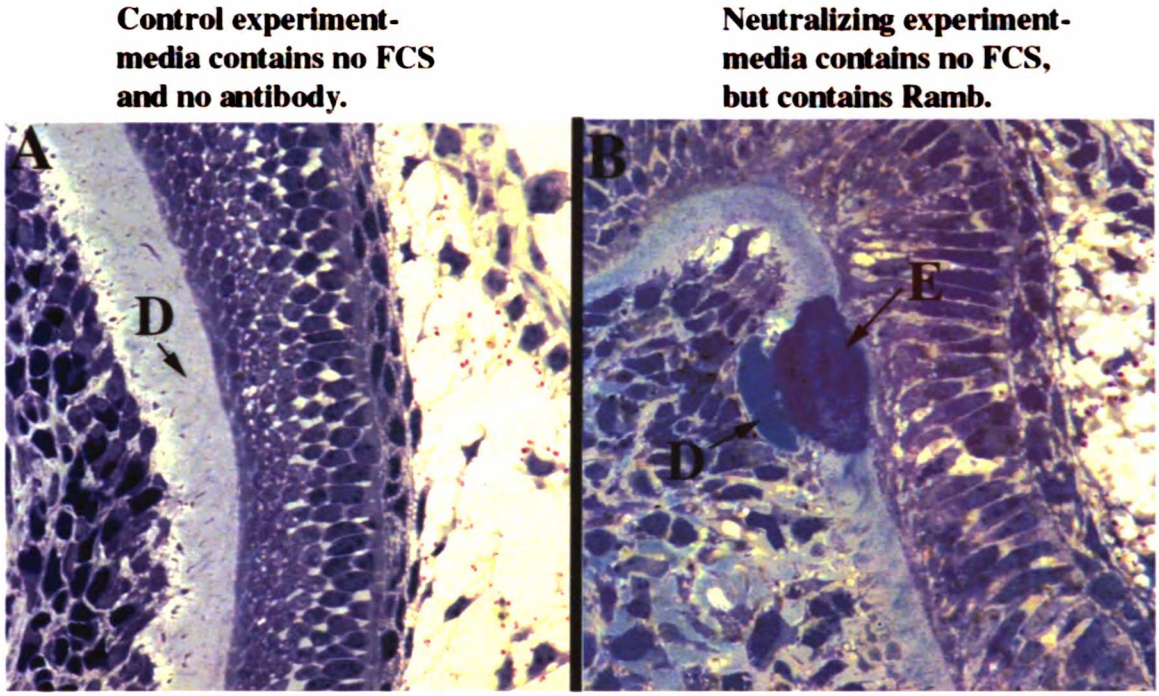


**Figure 5.**
**Tooth germ culture experiment in serum free media.**
(A) Control experiment shows no enamel formation at day 8 . (B) Neutralizing experiment shows initial enamel formation at day 8.

Enamel (E), and Dentin (D).

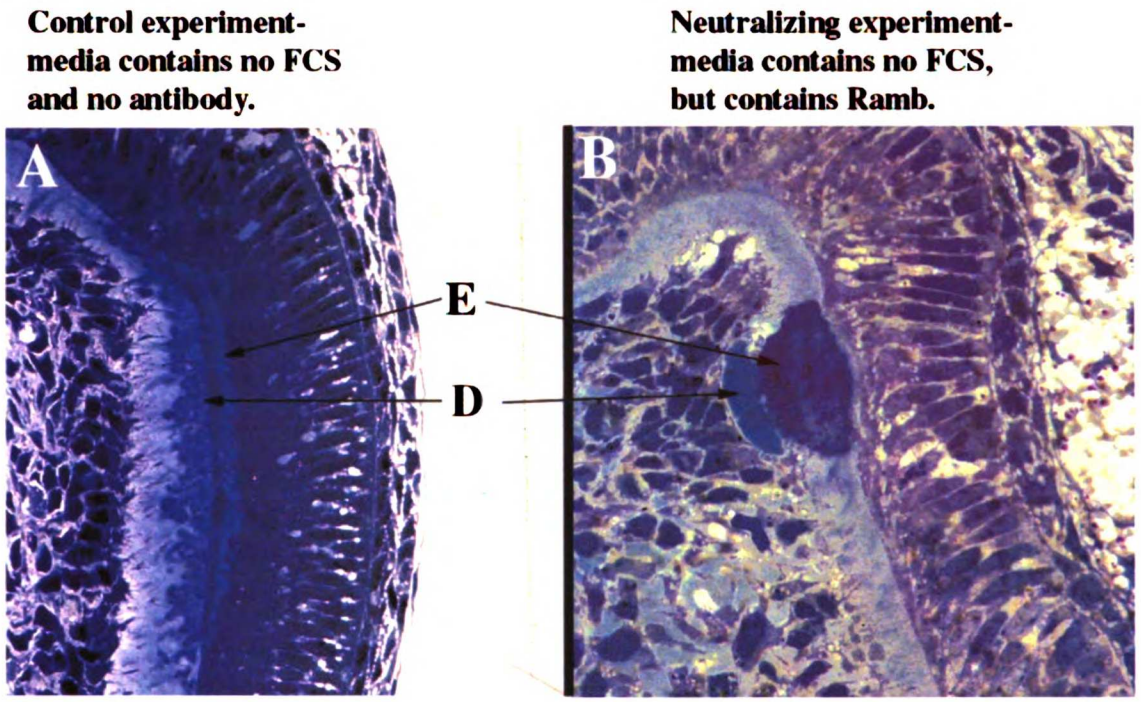


**Figure 6.**
**Tooth germ culture experiment in serum free media.**
(A) Control experiment shows initial enamel formation at day 12 . (B) Neutralizing experiment shows initial enamel formation at day 8.

Enamel (E), and Dentin (D).

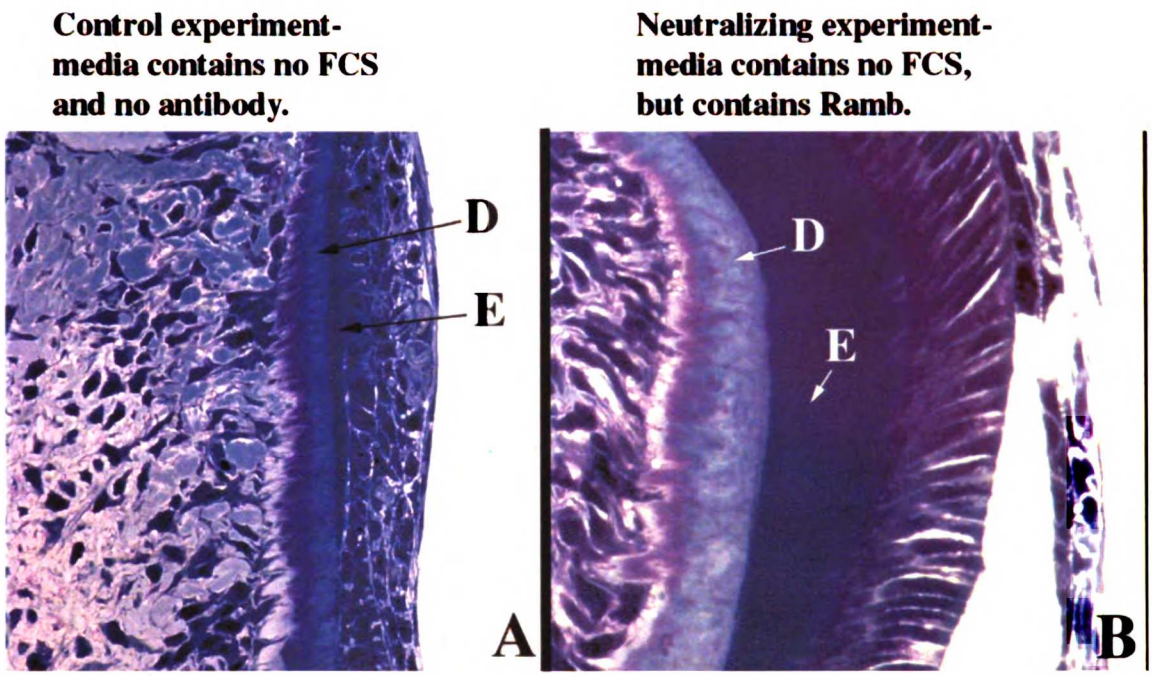


**Figure 7.**
**Tooth germ culture experiment in serum free media.**
(A) Control experiment at day 14 shows thin enamel formation without serum in the media. (B) Neutralizing experiment shows a thick enamel matrix at day 14, even without serum but in the presence of anti-ameloblastin antibody.

Enamel (E), and Dentin (D).

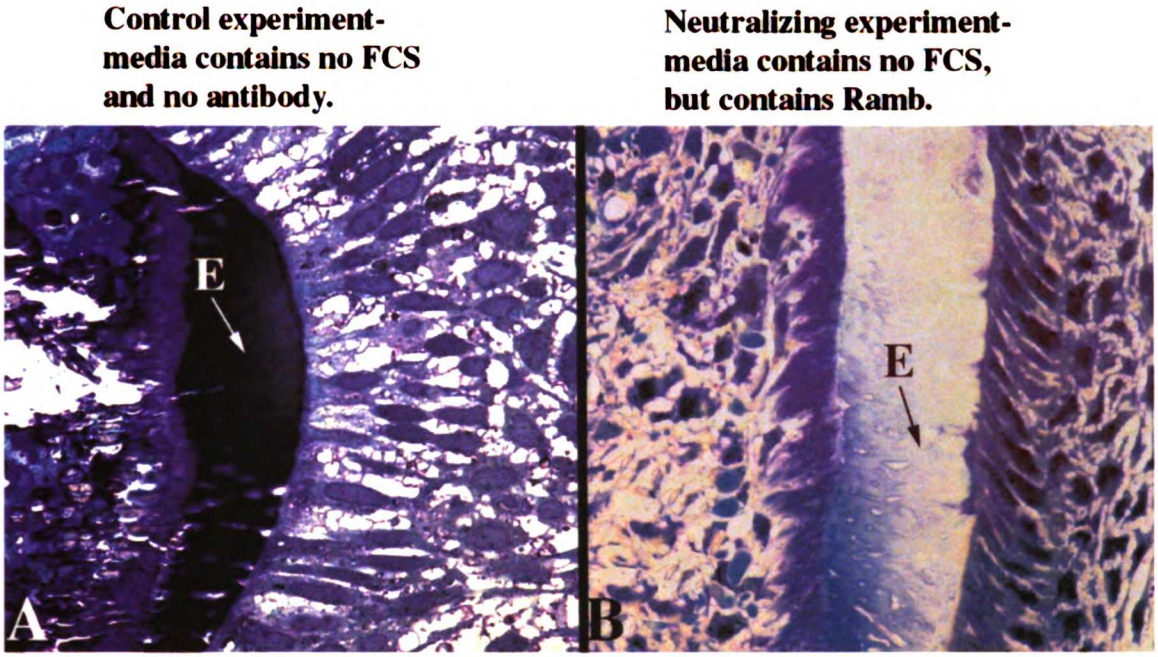


**Figure 8.**
**Tooth germ culture experiment in serum free media.**
(A) Control experiment shows a thick enamel matrix by day 16, that remains unmineralized. (B) Neutralizing experiment shows a well developed mineralized enamel matrix by day 18 in serum free, anti-ameloblastin antibody containing media.

Enamel (E), and Dentin (D).
Dark blue E = unmineralized enamel
Light blue and white E = mineralized enamel

## Chapter 4. Culture of primary "ameloblast like" cells from porcine tooth germs

### Pre-Introduction:

The start of the cell culture experiments led to a change in direction of my studies. Although my initial focus related entirely to the ameloblastin protein, I soon realized that the cell culture system was in its infancy and a lot of groundwork was necessary to move this science forward.

*Rationale*

Although this lab used a primary cell culture method leading to an immortalized porcine tooth germ derived cell line, the primary cell culture technique has not been fully established. Two major problems remained that affected my desired research. First, the immortalized porcine cells established in this lab do not produce ameloblastin [1], and later lost amelogenin expression (unreported observation). Second, in the primary cells, we could not consistently derive an epithelial like phenotype, without a predominant background of "other" cell populations.

*Scientific goals I expected to achieve with these experiment:*

I envisioned a number of short and long term goals when undertaking the challenging project of establishing a primary "ameloblast-like" cell culture system. First, was to establish cells that make ameloblastin that I would use for protein biochemical analyses. This would allow continuation of my work on this protein in a system novel to the field of tooth development. Second, was to produce a "pure" (single colony derived) primary population of cells to further analyze which cells produced specific enamel proteins

(including ameloblastin). Third, a long-term goal was to establish the phenotype of these "pure" cells in an *in vivo* environment.

## Introduction

Tooth enamel, formed by ameloblasts, is the hardest tissue synthesized in man, and other species. The synthesis is dependent on mesenchymal/epithelial interactions of different cells. These interactions rely on the communication between odontoblasts (mesenchymal) and ameloblasts (epithelial). The culture of odontoblast cells has been established, and proven invaluable in dissecting biochemical mechanisms behind dentin formation [2]. The culture of ameloblasts or epithelial cells from the tooth germs has been reported, however, these cells still remain difficult to culture, and little has been reported on the characterization of these cells.

Limeback was the first to report the culture of epithelial cells from a tooth germ [3]. A developing porcine tooth explant was utilized to initiate these cell cultures. He reported that the explants resulted in a co-culture of fibroblast and epithelial cell populations. The epithelial cells were shown to be positive to a tooth specific protein, amelogenin. This culture technique produced a mixed cell population, and further work using this technique was never completed.

MacDougall and coworkers, who described a mouse tooth germ cell culture system in DMEM media with serum, completed further work in this area. These reports described a mixed culture technique that also produced epithelial cells. These cells were shown to

be positive for amelogenin by RT-PCR [4]. These epithelial cells were also reported to produce mineralization nodules from the epithelial cells [5]. Although three abstracts were reported on this system, no paper was ever published, and thus much data are missing.

Kukita and coworkers were the first to publish the culture of tooth germ derived cells in a serum free media [6]. These culture conditions utilizing developing rat incisors also produced a mixed culture of spindle-like cells and fatter cobblestone epithelial cells. In contrast to Limeback's previous report the authors reported amelogenin expression in the spindle-shaped cells and not the epithelial cells through immunohistochemistry. They also reported that it was necessary to use collagen coated dishes to establish both primary and secondary cultures of these cells. Throughout the next three chapters I will be using the following nomenclature: when referring to spindle-shaped cells, SPABS will be used, and CABS for the cobblestone epithelial cells.

This lab has also established a primary culture system from porcine tooth germs [7]. Media similar to that described by MacDougall and coworkers was also shown to produce mineralizing nodules from the epithelial cells [5]. LHC9 media with serum was also used to produce a mixed cell culture system that was positive for ameloblastin and amelogenin by RT-PCR and immunohistochemistry [7]. When serum was removed from these cultures, the epithelial cells survived and the spindle-shaped cells began to be depleted. This led to further work with a similar media (LHC8e), which was used to select for epithelial cells that were later immortalized [1]. These immortalized cells were

entirely epithelial, and produced enamel proteins. However, primary cultures were not well established or characterized using this media.

The purpose of this study was to optimize cell culture from porcine tooth germs that produced tooth specific proteins. This optimization included the ability to select for epithelial (enamel protein producing) cells, and although this goal was achieved, there are some difficulties with this technique.

## Materials and methods

### Further establishing the primary culture of enamel organ derived cells from developing porcine molars:

*Dissection and cell culture* - Fresh porcine heads were obtained from the UCSF laparoscopy surgery center following routine use of the pigs for arthroscopy practice by the medical residents. Porcine impacted developing teeth were extracted after sterilizing the tissue with 70% EtOH and were placed in Hanks buffer. The enamel organ was then dissected from the tooth in Hanks buffer (without EDTA), washed with PBS and digested with collagenase/dispase in PBS (1mg/ml) for 1hour at 37°C. The digest was collected, centrifuged at 2500 rpm for 5 minutes and washed with PBS. Digestion was further performed with STV (0.05% trypsin, 0.025% versene, in saline A), for 5 minutes at 37°C. The cells were then centrifuged and washed as above, and 5 $X10^4$ cells were plated on a 100 mm Primaria (Becton Dickinson) tissue culture plate. The cells were cultured in MEM with 10% FBS, LHC8e media with 10% FCS, LHC8e media (Biofluids Inc., Maryland, USA) and in LHC8e serum free cultures.

*Cloning primary tooth germ derived epithelial cultures:*

Cells were first grown in the LHC8e alone or changed to LHC8e (10% FCS) after 2 weeks. One end of the cloning ring was first dipped into sterile vaseline, and placed around the colony of interest to isolate epithelial clones. The isolated clones were then digested in STV (0.05% trypsin, 0.025% versene, in saline A), for 10 minutes at 37°C, the digestion media was then inhibited with 0.5 mls of LHC8e (10% FCS). Each clonal cell suspension was then individually passed into a 6 well plate for growth. The media was changed the following day to LHC8e or LHC8e (10% FCS). The clones were then expanded using the same media following for further analysis.

*IHC:*

After primary digestion porcine tooth organ cells were plated in LHC8e media for two weeks, and then changed to media containing serum, (LHC8e (10% FCS)) for the following week. A PAP PEN (Zymed) was used to create 2cm circles on the bottom of a 100mm primaria dish. The cells were then passed into these circles in LHC8e media and allowed to settle for 24 hours. The cells were then fixed for 4 hours in 10 % non-buffered formalin and stored in 70% EtOH overnight. The cells were washed in PBS 5 minutes each three times. Goat normal blocking serum (15 ul in 1 ml PBS) was then added to the cells for 20 minutes at room temperature (RT). The excess serum was removed. Primary antiserum was diluted in PBS and the cells incubated at 37$^{o}$C for 30 minutes. The cells were washed in PBS for 5 minutes each 3 times. Diluted biotinylated secondary antibody in PBS was then added to the cells for 30 minutes RT. The cells were then washed in PBS, 3 times for 5 minutes each. Vectastain Elite ABC reagent was

made and allowed to sit for 30 minutes (Vectastain Kit, Vector Laboratories), then added to the cells for 30 minutes. The cells were then washed in PBS 3 times for 5 minutes each. The cells were then stained with a peroxidase substrate solution containing $NiCl_2$ and DAB for approximately 2 minutes (Vectastain kit). The immunostaining was observed by light microscopy (Nikon TMS inverted scope). Three different antibodies were used in this analysis. These antibodies were to ameloblastin [8], the extracellular calcium sensing receptor [9], and keratin [1].

*Total RNA isolation*

Total RNA was isolated using a S.N.A.P. total RNA isolation kit (Invitrogen, USA). Briefly, 150 ul TE, pH= 7.5 (containing 0.75ug/ml lysozyme) was added to 450 ul of Binding Buffer (lysis buffer). The cells were first washed twice in PBS and then the lysis buffer was added into a 100mm or 60mm cell culture dish. A cell scrapper was then used to pool the cells in the dish, and the cell/lysis mixture was transferred to a 1.5ml microcentrifuge tube. The mixture was passed through a 21 gauge sterile needle three times. The mixture was centrifuged at 13,000 rpm for 3 minutes and the supernatant transferred to a new microcentrifuge tube. 300 ul of isopropol was added to the supernatant and mixed by inverting 10 times. The solution was then centrifuged over a S.N.A.P. total RNA column (Invitrogen) for 1 minute at 10,000 rpm, RT. The column was washed 3 times with 1x RNA wash, and centrifuged dried. The total RNA was then eluted with Rnase free water and collected in a clean microcentrifuge tube. DNA was removed with the use of Dnase following the kit instructions. Total RNA was

determined with a UV-Vis spectrophotometer and precipitated with 0.15 volume of 2 M sodium acetate and 2.5 volume of 200 proof ethanol.

*RT-PCR*

Either mRNA or total RNA was used for RT-PCR reactions. The RNA was centrifuged at 16,000 g for 20 minutes at 4°C. The ethanol was aspirated and discarded 5-7ug of RNA was mixed with 1 ul of Oligo dT (500ug/ml) and the volume brought to 12 ul with Rnase free water. The mixture was heated to 70°C for 10 minutes and quick chilled on ice. The tube was then centrifuged and 4ul First Strand Buffer (Gibco), 2 ul 0.1 MDTT and 1 ul 10 mM dNTP mix was added to the tube. The tube was gently mixed and incubated at 42°C for 2 minutes. One ul of Superscript II (Gibco) was added by gently pipetting up and down. The reaction was then incubated for 50 minutes at 42°C and inactivated by heating at 70°C for 15 minutes. All heating reactions were done on a deltathermacycler (Ericomp, SanDiego). The cDNA was then used as a template in corresponding PCR reactions with amelogenin specific primers WU23 5' TTTTGCCATGCCTCTACCAC 3' and AMG04 5' CCCGCTTGGTCTTGTCTGTCG 3', and ameloblastin specific primers 5' GAAATGGCGGGAGGAAGAGGA labeled SU1 (upstream primer) and 5' GGGGTCATCAGTGGGTCAGCA labeled SL1 (downstream primer).

*Immunoprecipitation, SDS-PAGE and Western Analysis:*

*Antibodies* - Ameloblastin was immunoprecipitated from lysis buffer (150 mM NaCl, 1% Triton X-100, 0.5% NaDOC, 50 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 10%

glycerol and protease inhibitors) extracts, using the "ameloblast-like" cell line by immunoprecipitation with a 1/50 dilution of rabbit polyclonal antiserum Ramb (rabbit anti-bovine ameloblastin peptide). A Western blot confirmed the immunoprecipitation results, using a 1/100 dilution of chicken polyclonal antiserum Camb (chicken anti-bovine ameloblastin peptide). The antigen (YGAIFPGFGGMRPRL) for both the rabbit and chicken antisera was a synthetic peptide corresponding to the first 15 amino acids of the N-terminus of a 27- and 29-kDa ameloblastin protein fragment isolated from bovine enamel extracts [8]. A negative control was also done by loading approximately 30ug of protein from an SDS cell lysate of porcine cells grown in LHC8e + 10% FCS, and probing with a 1:500 dilution of IgG purified rabbit pre-immune serum.

*Immunoprecipitation* – Primary ameloblast cells were grown on 100 mm dishes. After the cells reached confluence, they were washed once with cold PBS, lysed with 0.5 ml of cold lysis buffer for 2 minutes and put on ice. The lysis mixtures were centrifuged at 13,000 rpm for 10 minutes at 4°C. The extracts were rotated on a tube rotator for 30 minutes at 4°C with 30 µl of a 50% slurry of protein A-sepharose beads to eliminate nonspecific protein binding. The mixture was centrifuged at 5000 rpm for 10 minutes 4°C and the supernatant was transferred to a new eppendorf tube with the Ramb antiserum and rotated for 1-3 hours at 4°C. Protein A-sepharose beads were then added to the solution and rotated for an additional 30 minutes at 4°C, centrifuged for 2 minutes at 5000 rpm, washed with lysis buffer, and centrifuged again. The protein A-sepharose immunocomplex was then washed twice more with buffer A (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.1% NP-40, 10% glycerol, 5 mM beta mercaptoethanol added fresh). The

depleted supernatant was removed by vacuum aspiration using a tuberculin needle. The purified ameloblastin protein was separated from the antibody complex by boiling in 2X SDS- sample buffer for 5 minutes, and chilled on ice.

*SDS-PAGE and Western blotting* - The immunoprecipitation mixture was then fractionated by SDS-PAGE using a 12% resolving gel. For Western blot analysis, proteins were transferred at room temperature (RT) to a nitrocellulose membrane for 1hour, blocked overnight in 5% nonfat dried milk/0.1% Tween-20 in tris-buffered saline (TBS), and washed 3 times with TBS. The membrane was then probed with chicken antiserum Camb (1/100) in TBS for 1 hour at RT. The membrane was washed 3 times for 5 minutes with TBS and incubated at RT for 1 hour with an affinity-purified rabbit anti-chicken IgG directed antibody conjugated to alkaline phosphatase, diluted (1/2000) in TBS. The membrane was then washed twice in TBS for 5 minutes and once briefly with sodium glycinate buffer (50 mM glycine, 8 mM $MgCl_2$, pH 9.6). The ameloblastin-antibody complex was detected by color reaction with 0.1% nitro-blue tetrazolium (NBT in 50% EtOH) and 0.05% 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

**Results:**

*General observation and technique development of the porcine tooth germ cell culture:*

Plating cells at different densities:

Cells were grown in three different media MEM(10% FCS), LHC8e(10%FCS) and LHC8e at three different concentrations/100mm dish ($1X10^6$ , $1X10^5$ , $1X10^4$). All media

at all concentrations produced epithelial clones. The cells grown in serum at a lower density ($1X10^4$) would establish epithelial clones, however they were taken over by the faster growing spindle-shaped cells. Cells plated at $1X10^5$ in serum media produced a culture with epithelial pockets surrounded by spindle shaped cells. The cells grown in serum plated at a higher density ($1X10^6$) showed the spindle shaped cells piling on top of the epithelial clones. The cells plated at a higher density established the epithelial colonies much faster, with a border of spindle-shaped cells wrapped tightly around the epithelial colonies. The epithelial colonies seemed to stop growing outward once the spindle-cells came in direct contact. Plating cells at higher densities ($1X10^6$) in LHC8e without serum led to healthier epithelial cells, with the lower density cultures establishing less epithelial colonies. Plating at high density for all culture conditions was very important in initially establishing primary cultures, especially with the serum free media.

Filtered versus unfiltered cells:

A sterile 2X2 gauze was used to filter the digested enamel organ cells to try and decrease the number of spindle-shaped cells. Although the cultured cells that were filtered would show epithelial colonies, the number of colonies was greatly diminished versus not filtering the cells through gauze prior to primary culture (figure 1). The filter was also reversed and washed to examine cells that did not pass through the filter, these cells, however did not attach to the dish.

Mechanical and different anatomical separation techniques were tried to optimize the epithelial cells:

Porcine tooth germ enamel organ was cut into small pieces with sterile scissors before enzymatic digestion showed the highest number of spindle-like cells and decreased epithelial cell populations. Enzymatic digestions were also tested on whole tooth germs, the enamel organ (soft tissue on top of the developing crown), and the developing crown. Separation of the enamel organ, before tissue digestion, established cell cultures with increased epithelial colony formation compared to digestion of the whole tooth germ or developing crown.

Media selection

*Further establishing the primary culture of enamel organ derived cells from developing porcine molars based on media selection (mixed cell culture):*

Cells cultured in MEM (10% FCS) showed a highly mixed cell culture. Cells cultured in LHC8e (serum free) were selective to some smaller spindly shaped cells, however this media produced more epithelial cell colonies. Cells cultured in LHC8e (10% FCS) also showed some of the smaller spindly shaped cells with some fatter cells showing ragged edges, however epithelial cells were observed. The cells cultured in LHC8e (10% FCS) revealed more of a mixed culture than LHC8e alone. (Figure 2.)

Of all three culture conditions LHC8e gave rise to the most epithelial cells with the least background of other cells, however, these epithelial cells were very slow growing, and after approximately 3-4 weeks the cells would become vacuolated and unhealthy (figure 3). These cells could be passed 2-3 times if a high density of cells was used, however the cells did not always reestablish colonization.

In contrast, epithelial cells grown in LHC8e (10% FCS) did not become vacuolated, and proliferated faster. The "non-epithelial like" cells also proliferated faster in serum conditions encircling the epithelial colonies (figure 4). The epithelial cells grown in serum conditions could grow for a few months, and did not die at confluence. When these cells were passed, it was difficult for the epithelial cells to re-establish colonies, some epithelial colonies would initially form after the first couple of passages, but the culture would eventually become almost entirely spindle-shaped cells.

I determined that LHC8e with and without serum gave the best results for optimization of the epithelial cells. The problem, however, of a predominant background of other "non-epithelial" like cell types in serum containing media, while cells cultured in serum free media were difficult to pass, still remained.

*LHC8e initially and then change to LHC8e (10% FCS).*

To utilize the selection advantage of LHC8e media, cells were first grown in LHC8e for approximately 2 weeks and then transferred to LHC8e(10% FCS) to utilize the proliferation potential of this media. The results observed in these experiments were similar to those cells that stayed in the same media. Initially more epithelial cells were selected and grew, however with the addition of serum, the other faster growing "non-epithelial like" cells took over the dish population. Some dishes were also transferred back to serum free media (LHC8e), however this media would not support the long-term growth of the epithelial cells.

*Cell selection through cloning rings:*

I had established the ability to consistently grow pockets of epithelial cells, however they were very difficult to pass without serum, and culturing with serum led to high backgrounds of cells that we were trying to select against. The epithelial clones grown in LHC8e were isolated by cloning rings, trypsin digested and passed into dishes. The problems remained with these colonies as found in previous cell cultures. Isolated colonies transferred to LHC8e and LHC8e (FCS) did not pass well into another dish, limiting further colonization.

*Establishing the mRNA and protein expression of the porcine tooth germ derived mixed cell culture system:*

*RT-PCR*

The cDNA isolated from the mixed cell population confirmed the presence of amelogenin mRNA with a positive PCR product of 550 base pairs. This same product was confirmed in a positive control using a porcine tooth germ developmental library. A negative control was done with no template, but all other PCR reagents, confirming the specificity of the amelogenin signal to the isolated cellular cDNA. (Figure 5).

*IHC*

Cultured cells were passed into a dish for immunohistochemical analysis. Porcine tooth germ derived cells stained positive for ameloblastin, Ca-sensing receptor, and keratin. The dilutions were as follows: 1:500 dilution of the primary antibody was used in the experiments with anti-ameloblastin antibody and anti-calcium sensing receptor antibody,

1:10 dilution with the anti-keratin antibody. A negative control experiment using a 1:500 dilution of pre-immune serum revealed no staining. (Figure 6).

*Immunoprecipitation:*

Cells were cultured in LHC8e for one week and then changed to LHC8e+ (10% FCS) per materials and methods. The cell lysis was first examined by SDS-PAGE revealing a large amount of denatured protein at approximately 40-60 kD. This extract was shown to contain many different fragments that stained positive for the ameloblastin antibody including the 27-, and 29-kD fragments that this peptide antibody was derived from (see chapter 2, and 3 for details) Other positive stained fragments were between approximately 32-43 kD, and a larger fragment at 60 kD. Rabbit peptide anti-ameloblastin antibody (Ramb) not immunoprecipitated was also stained with chicken anti-peptide ameloblastin antibody (Camb) as a negative control to confirm that the antibodies alone did not have cross reactivity. (Figure 7). A negative control was also completed using a rabbit pre-immune serum on the cell lysate showing negative staining of any specific bands.

**Discussion:**

In these studies I established experimental conditions for the primary mixed culture of porcine tooth germ cells. This optimization included enzymatic digestion, non-filtering of the cells, plating densities and mechanical separation of the enamel organ from the

crown. I chose to optimize conditions to select for cells with a cobblestone epithelial phenotype because, earlier studies completed in this lab established an immortalized epithelial cell population that proved to be positive to signals associated with tooth specific cells [1].

A two-tiered approach of enzymatic digestion (collagenase/dispase followed by trypsin) proved beneficial for the optimization of increased epithelial colony formation in culture. The use of trypsin only for cell and tissue digestion from primary tooth germ cultures has been reported previously. In this first published study on tooth germ culture, whole organs were put into a culture dish, and the primary cell outgrowths were trypsinized to establish monolayer cultures [3]. Epithelial cultures from mouse tooth germs has also been reported by "epithelial separation from the dental papilla with 1% trypsin", followed by a 0.25% trypsin digestion of the epithelial cells, however, this protocol was published in an abstract form and therefore it lacked many details [10]. The use of collagenase/dispase (without trypsin) as a cell digestion protocol has also been reported in the literature for the establishment of primary and immortalized porcine "ameloblast-like cells" [1, 7]. A dual enzyme approach has also been reported in the culture of cells from the tooth germ, with first collagenase or dispase followed by trypsin digestion [6, 11]. I found that the use of both enzymes, first collagenase/dispase (mixed) followed by trypsin digestion (details in materials and methods) increased the number of epithelial clones in the mixed culture system.

Other approaches leading to increased epithelial clones in the mixed culture system included non-filtering of the cells. Initially the cells were filtered to get rid of larger cellular debris and potentially select for the epithelial cell phenotype, however it was shown that epithelial cells were not selected for by filtering. Enzymatic digestion has shown the ameloblast cells to be released in sheets [6], although these cells are later further digested by trypsin, it is possible that some of these cells are still adherent and not allowed to pass through the filtration mechanism. Although, the filter was washed to try and culture the larger cells, this method was not as successful as culturing enzymatically digested tissue. The protocol was then changed to culture conditions that did not include cell filtration.

Mechanical removal of the enamel organ before enzymatic digestion also proved important to increase epithelial colony formation in the mixed culture system. The epithelial cells most likely producing the enamel specific proteins are the ameloblasts, hence "ameloblast like cells"[1]. This ameloblast cell layer lies underneath the enamel organ, with the cells adjacent to the developing enamel. The enamel organ was carefully peeled off at the junction of the developing enamel, likely exposing the ameloblasts for further digestion. The enamel organ, the developing enamel, and a whole tooth germ were enzymatically digested. Tooth germs mechanically digested with sterile scissors were also used as a comparison. The digested enamel organ, previously removed, led to an increase in epithelial colony formation. Earlier literature supported the culture of whole porcine tooth germs to establish initial epithelial colonies [3], or the removal of a cell layer from incisor rat dentin [6]. Previously in this lab, both mechanical separation

with scissors and digestion of the removed enamel organ had been used [1, 7] (personal communication, Cen Goa). In one culture the enamel was dissected from the tooth, and placed in a culture dish containing collagenase/dispase to determine if the ameloblast layer remained adherent to the developing enamel. After plating some epithelial colonies did form indicating that some ameloblasts likely adhere to the enamel layer when the enamel organ is mechanically removed.

Finally, cell density dependence was determined to influence the number of successful epithelial colonies. Little data is reported in the literature, regarding initial cell counts to establish a successful primary tooth germ culture system. MacDougall and co-workers did report 5 X $10^5$ cells/16mm well to establish cultures from developing mouse molar tooth germs [10]. Cell density played an important role in successfully establishing epithelial colonies. When the cell cultures were initially plated, fewer epithelial cells were seen compared to the spindle shaped cells. In serum conditions it seemed important to reach a critical mass of epithelial cells to establish these colonies ($1X10^5$). However, if the cell density was too high, the spindle shaped cells quickly took over the cell culture dish, not allowing the epithelial colonies to form. In a serum free culture condition (LHC8e), it was important to start with a higher concentration of epithelial cells ($1X10^6$), using media selection to select against the spindle-type cells.

In trying to establish a consistent primary culture system using the media from the materials and methods I was left with a few challenges. Although the cultures containing serum gave rise to pockets of epithelial cells, the dishes contain highly mixed cell

populations. When we culture without serum, colonies of epithelial cells also form as they did in serum containing media, with less background of non epithelial like appearing cells. However, these cells are much slower growing, and over time became vacuolated in appearance. The cells in the serum free media do not pass well, and although the serum containing cells do pass, the epithelial phenotype is rarely established as clonal pockets among the mixed cell population. It is possible that the "non-epithelial like" cells quickly establish and overcome the epithelial like cells. It is also possible that there is a problem with cell adherence. This hypothesis is further supported in trying to establish the epithelial phenotype using cloning rings. Clear populations of epithelial cells when isolated, digested, and passed using a cloning ring did not adhere to the dish and reestablish epithelial colonies. Kukita and coworkers found that without collagen coated plates, secondary cultures containing epithelial cells would not form [6]. We had established the use of Primaria (Falcon) dishes, a positively charged plate that helped with our initial cultures [7]. These plates, however, did not always help in further culture of epithelial cells.

The primary culture of a non-mixed epithelial "ameloblast like cell" has not been completed in the past. There have been some attempts to culture ameloblast-like cells, however, the cell populations always remained very mixed [7] [3, 6, 10]. The rationale behind using different media was to try and find a media that was able to select for enamel organ derived cells depicting an epithelial phenotype. When the porcine enamel organ was cultured in different media containing serum the culture gave rise to 3 cell types: spindle-type cells, epithelial-like cells with a ragged edged appearance, and an

epithelial cells with smooth borders. The spindle and smooth edged epithelial cell types had been reported previously [7] [3, 6, 10]. This is the first report to describe the ragged edged epithelial type cell. Further studies are necessary to isolate this cell type to determine if it is a cell type specific to tooth germs.

I observed that when the porcine enamel organ was cultured in serum free media (LHC8e), cobble stoned shaped epithelial cells were selected for. In three-four weeks of growth these cells would slowly proliferate, and then the cells would become larger and vacuolated, and finally stop cell proliferation. These cells were also very difficult to pass. I then tried to use the serum free media to select for epithelial cells, and then after selection of one week, change to a serum media (LHC8e +10% FCS) to rely on the growth factors to support the epithelial cells. The culture conditions of a serum free to serum conditions did produce a viable cell population. These cells were further shown through immunohistochemistry to produce ameloblastin, keratin, and a Ca-sensing receptor. This protein pattern establishes the cells as an epithelial derived (keratin +) tooth specific (ameloblastin +) population. This pattern is not surprising considering that similar results have been reported when the cells grown in this media were immortalized [1]. The Ca-sensing receptor, however, had not been previously reported in the literature, this led to co-authorship on paper describing this receptor in tooth germ cells [9]. These initial culture conditions of serum free selection, and then serum growth worked well for the establishment of proliferating cells including epithelial colonies, however, these conditions could not be used to completely select for a single cell type. The mixed culture, however, was positive for tooth specific signals (amelogenin mRNA) using PCR.

Further confirmation that these cell populations produced enamel proteins (ameloblastin) was completed through immunoprecipitation. One report had previously used Western analysis with an anti-amelogenin antibody to characterize tooth germ derived cells from a porcine tooth explant [3]. This is the first study to report the immunoprecipitation of enamel matrix proteins from cultured cells. Not only did I see a large ameloblastin positive band of approximately 60 kD, but numerous bands between 40-32 kD. These 32-40 kD bands are likely intermediate degradation products of ameloblastin. The 27 and 29 kD protein (the fragment that this protein was made to) could also be observed [8]. The same pattern of ameloblastin immunopositive staining has been reported in the literature using a peptide made against an antibody approximately 100 amino acids downstream from our antibody sequence [12]. The negative control also revealed two bands of 50 and 43 kD. These bands were artifact staining of the antibody used in immunoprecipitation, and was confirmed by staining with secondary antibody only (data not shown). Further characterization of these enamel proteins using cell culture will prove to be a valuable tool in this field.

**Conclusion**

The ability to culture tooth germ derived cells that produce enamel proteins is an essential tool to move this field forward. This study reports the optimization of cell culture conditions to establish cells populations that produced enamel proteins from a mixed culture. It also reports some of the difficulties in establishing a non-mixed primary cell culture. Using LHC8e media alone, will produce mostly epithelial cells, however further

passage of these cells is difficult, therefore initial studies can be done on these primary cultures without passage. Using this same media we can select for epithelial cells that become more vibrant in a serum containing media, these cells have been shown to produce enamel, however mixed cultures also form. Depending on the areas of interests these techniques will prove useful for analysis of tooth development in this field. Characterization of primary cell populations has been completed in Chapters 6 and 7.


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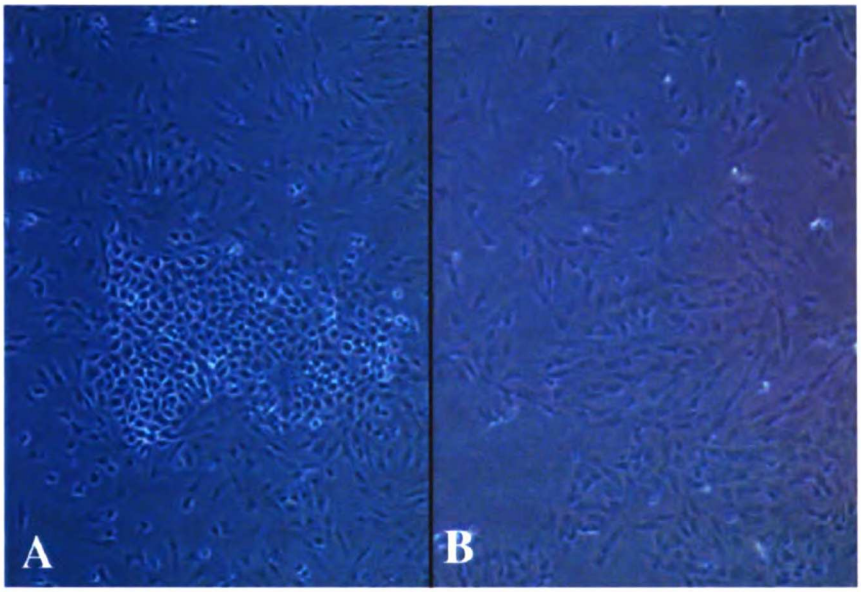



**Figure 1.**
**Phase contrast images of porcine tooth germ derived cells grown in LHC8e with 10% FCS.**
(A) Cells were grown according to materials and methods, and were not filtered before primary culture. (B) Cells were first filtered through a sterile 2X2 gauze in an attempt to select for the cobble stone shaped epithelial cells. The spindle shaped cells were selected instead.

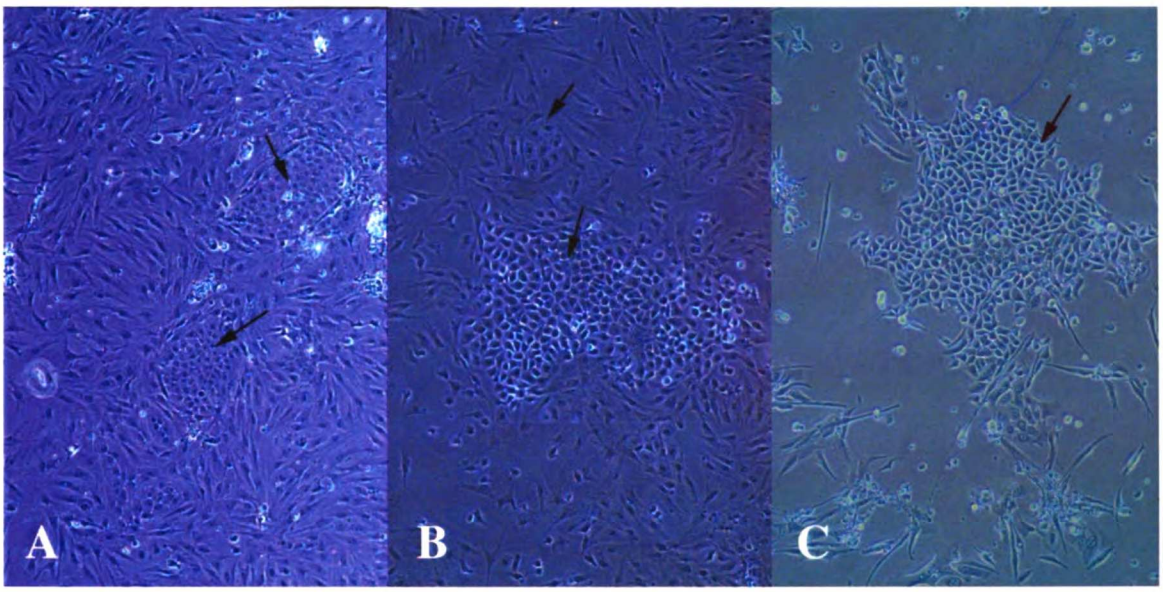



**Figure 2.**
**Phase contrast images of porcine enamel organ derived cells grown in different media (10X).**
(A) Cells grown in MEM with 10% FCS show a mixed culture with spindle shaped cells and cobble stone shaped cells. (B) Cells grown in LHC8e with 10% FCS show a similar mixed culture of the spindle shaped cells and cobble stone shaped epithelial cells. (C) Cells grown in LHC8e without FCS select for cobble stone shaped epithelial cells with a decreased background of spindle shaped cells versus cells selected for in serum containing media. Arrows show the cobble stone shaped epithelial cells.

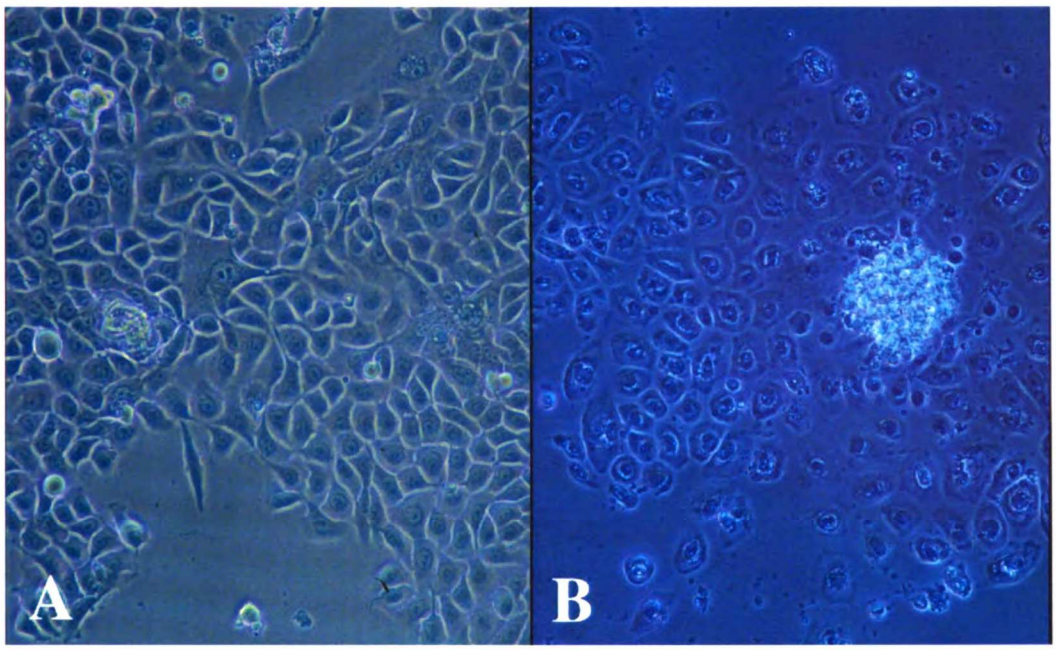



**Figure 3.**
**Phase contrast image of porcine derived tooth germ cells grown in LHC8e (20X).**
(A) Cells were grown for approximately 2 weeks in LHC8e. (B) Cells kept in the same media eventually become vacuolated and unhealthy in LHC8e after approximately 3 weeks.

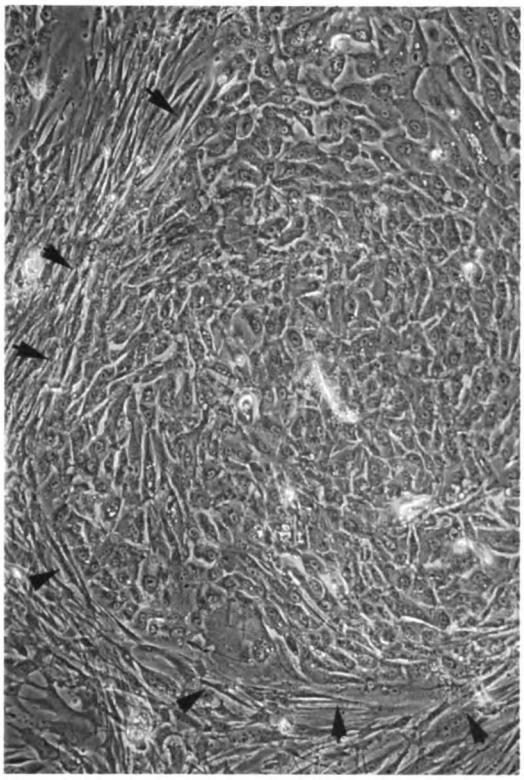
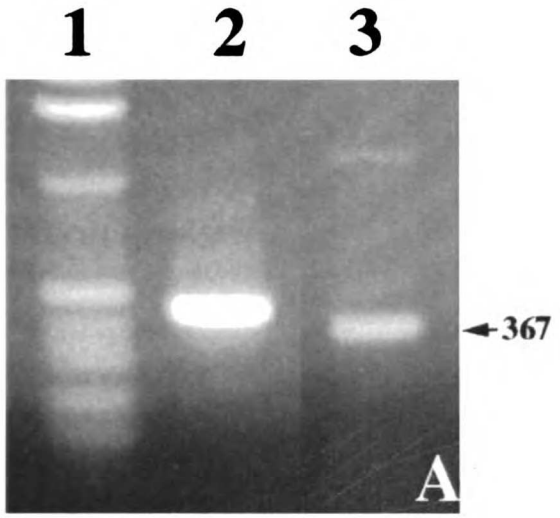


**Figure 4.**
**Phase contrast images of porcine enamel organ derived cells grown in LHC8e with 10% FCS (10X).**
The culture was allowed to come to confluence. The cobble stone shaped epithelial cells are surrounded by the spindle-shaped cells in this mixed culture. Arrowheads point to the spindle-shaped cells that are perpendicular to the epithelial colony.



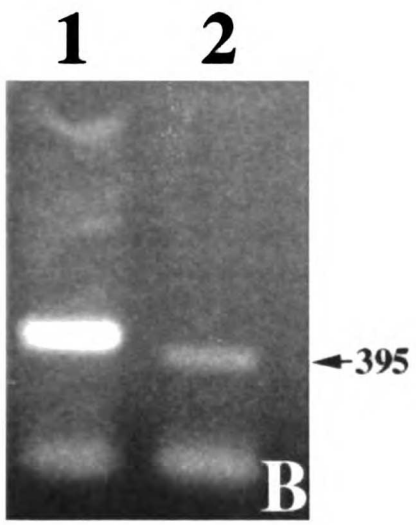




**Figure 5.**
**PCR of porcine cells grown in LHC8e + 10% (FCS).**
**A)** Gel electrophoresis of PCR products using ameloblastin specific primers.
(Lane1) Marker. (Lane 2) G3PDH control at 395 base pairs. (Lane 3) cDNA isolated from porcine tooth germ cells grown in LHC8e + 10% (FCS). Correct size is 367 base pairs shown with arrowhead using porcine ameloblastin specific primers.

**B)** Gel electrophoresis of PCR products using amelogenin specific primers.
(Lane 1) G3PDH control at 395 base pairs. (Lane 2) cDNA isolated from porcine tooth germ cells grown in LHC8e + 10% (FCS). Correct size is 395 base pairs shown with arrowhead using porcine amelogenin specific primers.

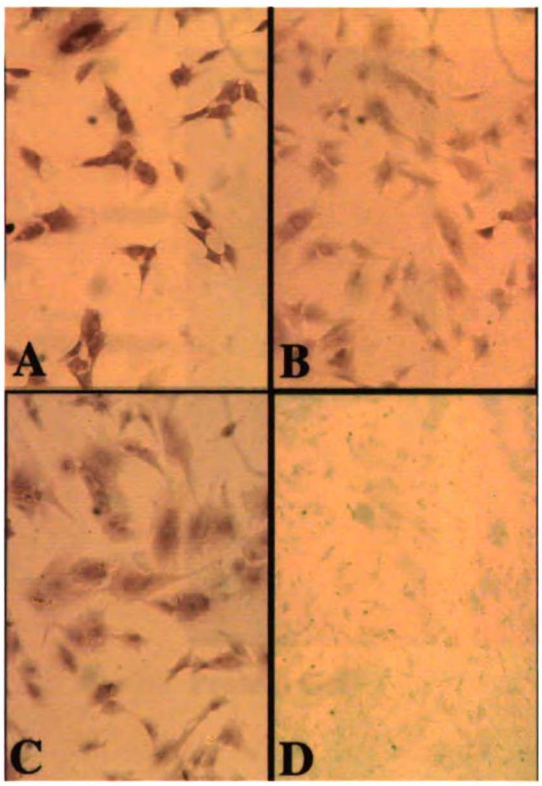



**Figure 6.**
**IHC of porcine cells grown in LHC8e and then changed to LHC8e + 10% (FCS) (20X).**
(A) Ameloblastin stained positive with a 1:500 dilution of Ramb. (B) Keratin stained positive with a 1:10 dilution of anti-keratin antibody. (C) Calcium sensing receptor stained positive with a 1:500 dilution of anti-peptide calcium receptor antibody. (D) Rabbit pre-immune serum was shown to be negative.

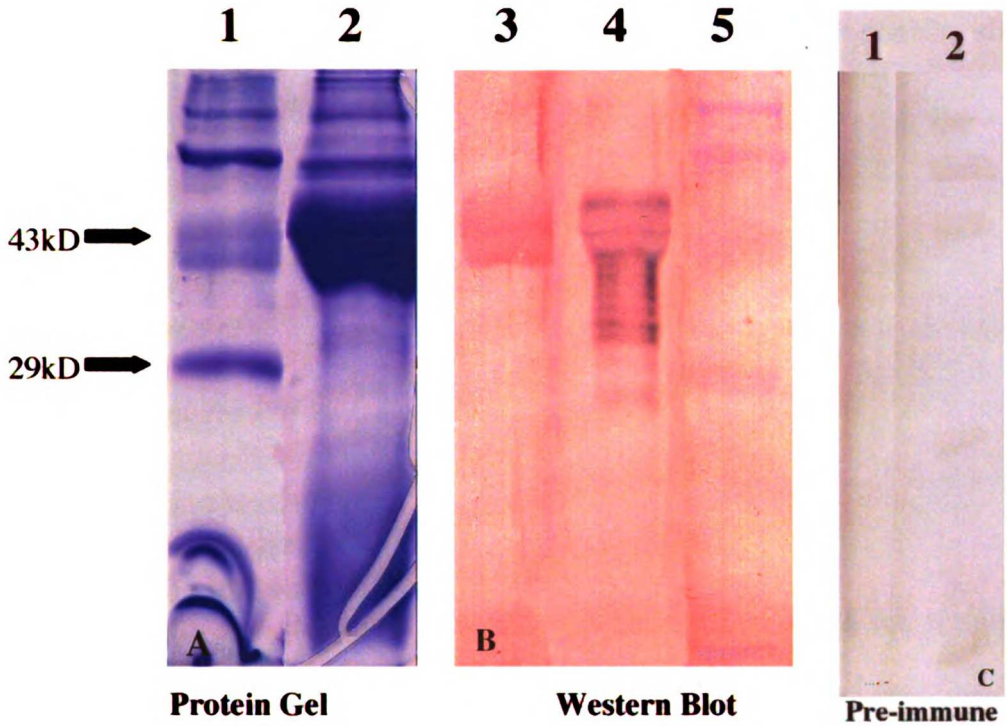



**Figure 7.**
**Western blot analysis of porcine enamel organ derived cells.**
(A) SDS-PAGE of immunoprecipitated cells using ameloblastin peptide antibody Ramb. (Lane 1) Protein marker. (Lane 2) Immunoprecipitate.
(B) Western blot of Ramb (rabbit anti-bovine ameloblastin) immunoprecipitated cells probed with a different anti-ameloblastin antibody (Camb). The Camb antibody was made using the same peptide, however it was made in chicken.
(Lane 3) Ramb antibody ran by itself as a control, shows minor cross reactiviy, likely due to recognition from the anit-chicken secondary antibody. (Lane 4) Immunoprecipitate showing different fragments of ameloblastin in the cells. (Lane 5) MW markers.
(C) Western blot of porcine derived tooth germ cells with rabbit pre-immune serum. (Lane 1) Pre-immune serum shows no specific staining. (Lane 2) Molecular weight markers.

## Chapter 5. Primary culture and characterization of cobble stone shaped "ameloblast like" cells from human tooth germs

### Pre-Introduction

I characterized primary human ameloblast-like cells from human tissue and report these results in chapters 5 and 6.

*Why change systems?*

I initiated my cell culture studies using porcine tissue, with the long-term goal of establishing this system using human tissue. Developing porcine teeth are readily accessible through tissue sharing facilities at UCSF, therefore I could do a lot of technique development before moving into the human system. I was interested in developing a culture system using human tissue for future work in tissue engineering utilizing the cell culture system (we were concurrently making a human tooth development library which would facilitate these studies). Although, the porcine system was useful, I came to a scientific crossroads. This scientific crossroad included the completion of a human tooth germ cDNA library and the appreciation that though I was working with proteins and cDNA from three different species, my work would be most effective if I focused on one system. The ameloblastin gene had been analyzed from a bovine tooth development library (Chapter 2), however, bovine tooth germs were not readily accessible, although access to bovine enamel protein extracts was. To continue work with this gene and protein, I decided to switch to a human system using my initial cell culture information from porcine tooth germs. I identified a human ameloblastin

cDNA through PCR, and further successfully established a primary human cell culture system (Chapter 5 and 6).

**Chapter 5 Establishing a primary human epithelial tooth germ derived cell line with ameloblast characteristics.**

Rationale: The culture of enamel organ epithelial cells was first established using porcine tissue, however in order to fully exploit this new technique in various studies I felt it was important to establish a human counterpart. This would not only confirm the repeatability of my newly established technique, but allow further techniques related to the study of human disease such as ameloblastomas, and the replacement of lost tissue with tissue engineering techniques, as well as understanding the basic mechanisms behind tooth development in an *in vitro* human model. This technique was further modified to examine specifically what cell types from a mixed culture were responsible for expressing tooth specific proteins.

**Introduction**

As the oral cavity forms during embryonic development the epithelial lining begins to form a thickened band (primary epithelial band) from which the teeth will develop in the alveolar arches. This epithelial band gives rise to two subdivisions of cells: the vestibular lamina and the dental lamina. The vestibular lamina forms as a result of proliferation into the ectomesenchyme, and eventually gives rise to the vestibule between the cheek and

tooth-bearing area, where as the epithelium with in the dental lamina continues to proliferation locally. The local epithelial proliferation in the dental lamina gives rise to ingrowths into the underlying ectomesenchyme. These epithelial ingrowths are not only derived from the same keratinocyte cell layers that give rise to the buccal mucosa (buccal infoldings), but they correspond to the positions of the later forming teeth [1]. What signaling factors lead to the further differentiate of epithelial cells into keratinocytes that form the buccal mucosa or tooth forming cells is still poorly understood. Calcium, however has been shown to be a key regulator of keratinocyte differentiation and proliferation.

In vitro studies of keratinocytes in low calcium concentrations in media (0.05-0.10 mM) lead to higher keratinocyte proliferation, widened intercellular spaces, and lack of stratification [2]. Keratinocyte terminal differentiation can be induced by the addition of 1.2 mM calcium into the media leading to cell stratification and desmosome formation [2]. Calcium induced differentiation is associated with tyrosine phosphorylation, likely triggered from an extracellular calcium sensing receptor [3, 4]. This same calcium receptor has recently been found in ameloblasts from tooth germs (Chapter 4, [5]). This receptor could lead to a similar control of ameloblasts as found in keratinocytes through external calcium levels in the media.

Further understanding of factors, such as calcium, that control differentiation and proliferation cellular events, requires a primary epithelial tooth germ cell culture. There is little information existing on the culture of tooth derived epithelial cells (See

introduction Chapter 5). Although there have been some reports, they all report the culture of a mixed cell population [6-10], unless the cells have been immortalized through SV-40 transfection [11, 12]. The use of a primary culture system eliminates any alteration of critical regulatory cellular events brought about by SV-40 transfection.

Our lab has been instrumental in pioneering the culture of porcine ameloblasts leading to an immortalized ameloblast like cell line [12]. However, there are some shortcomings with our newly developed technique. These shortcomings were mostly related to the culture of a mixed cell population in a serum environment, as well as not being able to study the effects of differentiation on the immortalized cells. Therefore, I first used porcine cells to optimize a primary cell culture technique (Chapter 4), followed by application of this technique to human enamel organ epithelial cells described in this report. In this present study it was my goal to establish and characterize primary human tooth derived epithelial cells, and further examine the role calcium plays in these ameloblast-like cells development.

## MATERIALS AND METHODS

Differential selection of the cobble-stoned appearing epithelial cell type

*Media selection - Application of cell culture technique to human tooth germs for selection of primary epithelial enamel organ derived cell types:*

All the protocols involving the human tissue specimens were approved through the UCSF committee on human research. Tooth germs were isolated from human abortus specimens at an average age of 18-21 weeks. The initial specimens were disrupted and

nonviable through a D and C suction technique. The tooth germs were isolated, washed in PBS, and digested with collagenase/dispase in PBS (1mg/ml) for 1hour at 37°C. The tissue/cell mass was then collected, centrifuged at 2500 rpm for 5 minutes and washed with PBS. Digestion was further performed with STV (0.05% trypsin, 0.025% versene, in saline A), for 5 minutes at 37°C. The cells were then centrifuged and washed as above, and 5 $X10^4$ cells were plated on a 100 mm Primaria (Becton Dickinson) tissue culture plate. Cells were cultured in LHC8e media, with and without fetal calf serum (FCS), and supplemented keratinocyte media (KGM-2) with and without serum, as well as different calcium concentrations (0.05, 0.075, 0.15, 0.25, 1.0mM). The culture conditions used are summarized in the table below, along with the specific analysis completed at each condition. These methods are described in detail as follows.

| Media | FCS | $Ca^{2+}$ (mM) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | |
| | | | LMx | TEM | FACS | Adhesion | Prolif. | RT-PCR | IHC | Western |
| LHC8e | | | x | | | | | | | |
| LHC8e | 10% | | x | | | | | | x | |
| | | | | | | | | | | |
| KGM-2 | 10% | 0.05 | x | | x | | | | | |
| KGM-2 | 5% | 0.05 | x | | | | | x[1,2] | | |
| KGM-2 | 2% | 0.05 | x | | x | | x | | | |
| KGM-2 | | 0.05 | x | x | x | x | x | x[1,2,3] | x[1,2] | x[1,2] |
| KGM-2 | | 0.075 | x | | | | | | | |
| KGM-2 | | 0.15 | x | x | | x | x | x | | |
| KGM-2 | | 0.25 | x | | | | | | | |
| KGM-2 | | 1.0 | x | x | | | x | x[1,2] | x | |

**Table 1**
Chart of media used to establish selection of cobble-stoned shaped epithelial cells. Included are the different experiments performed on the cells grown in different media. Light microscopy (LMX). Transmission electron microscopy (TEM). Fluorescent activated cell sorting (FACS). Proliferation assay (Prolif.). Immunohistochemistry (IHC). Western blot (Western).

1- ameloblastin
2- amelogenin
3- enamelin

*Light microscopy:*

Cells were grown in different media conditions (listed in table 1). Cells were also grown in three dishes containing KGM-2 with 0.05 mM calcium media. At approximately 70% confluence the media was changed to the following calcium conditions: 0.05, 0.15, or 1.0 mM $Ca^{2+}$. The cells were then analyzed by light microscopy for intercellular bridge formation over a periodic time course of 1 hour to 5 days. A Nikon TMS inverted scope in phase contrast was used for light microscopy.

*Transmission electron microscopy:*

Cells were grown in KGM-2 + 1.0 mM $Ca^{2+}$. Cells were fixed in 3% paraformaldehyde-2.5% glutaraldehyde overnight, and washed in PBS. The cells were immunostained using a 25-kDa anti-bovine amelogenin antibody and examined by TEM. Cells grown in the different calcium concentrations described above (0.05, 0.15, or 1.0 mM $Ca^{2+}$), were also processed and examined by TEM.

*Shape selection of cell types*

*Transfection of cells with SV40:*

Human cobble-stoned shaped epithelial "ameloblast-like" cells (CABS) were selectively grown in serum-free KGM-2 + 0.05 mM $Ca^{2+}$ and transfected at primary culture with SV-40. Cells were grown to 75% confluence. Two-three hours before transfection fresh media was added to the cells, and the cells were transfected with linearized SV-40 [12] by $CaPO_4$ DNA precipitate. This precipitate was prepared by mixing 210 ul of sterile

pyrogen free $H_2O$ with 25ul of .22 um filtered 2.5 M $CaCl_2$ and 15 ul of DNA (1ug/ul). This $CaCl_2$ mixture was added dropwise to 250 ul 2X Hepes-buffered saline HBS, while bubbling air through the solution. This solution was allowed to sit for 30-40 minutes. Approximately 5 minutes before adding the precipitate to the cells 5 mls of DME:$H_2O$ (1:1 ratio) was added to the 100mm dish of cells. The 0.5 ml precipitate slurry was then added to the cells, and gently swirled before further incubation for 3 hours at 37$^0$C. The dish was then washed approximately 15 times with 5 washes of PBS followed by 10 washes of KGM basal media. Growth media (KGM-2 + .05 mM $Ca^{2+}$) was then left in the dish and changed every 24 hours for 7 days.

*Clonal isolation of SV-40 transfected cells with cloning ring:*

Individual clones of human SV-40 transfected cobble-stoned shaped "ameloblast-like" (CAB) cells were isolated at passage 4 with the use of a sterile cloning ring. One end of the cloning ring was first dipped into sterile vaseline, and used to isolate epithelial clones. The isolated clones were digested in STV (0.05% trypsin, 0.025% versene, in saline A), for 10 minutes at 37°C, and the digestion media was then inhibited with 0.5 mls of KGM-2 +2% S + .05 mM $Ca^{2+}$. Each clonal cell suspension was then individually passed into a 6 well plate for growth. The media was changed the following day to KGM-2 + 0.05 mM $Ca^{2+}$ after cell attachment. The clones were then expanded following previous cell culture procedures for further analysis.

*FACS analysis:*

Fluoresce activated cell sorting (FACS) was performed to examine the ability to separate the predominant two cell types of CABS and spindle shaped cells (chapter 4) from a mixed culture. A primary human derived tooth germ mixed cell culture (containing two predominant cell types: CABS, and spindle shaped cells grown in KGM-2 + 10% FCS + .05 mM $Ca^{2+}$) was analyzed by FACS.

The second experiment utilized two isolated cell types as controls (CABS and spindle shaped cells). These two cell types were analyzed alone and mixed with one of the cells (CABS) labeled by a fluorescent cell marker (cell tracker green CMFDA). This experiment utilized three different samples. The first sample, cells that had been grown in KGM-2 +2% FCS + 0.05 mM $Ca^{2+}$ to select for the spindle shaped cell type, was analyzed by FACS. The second sample, a SV-40 transfected epithelial cell strain (clonally isolated CABS), was labeled by cell tracker green CMFDA (5-chloromethylfluorescein diacetate, Molecular probes) and analyzed by FACS. The third sample contained unlabeled spindle shaped cells mixed with fluorescent labeled CAB cells. All sample cultures were digested with STV for 5 minutes, centrifuged for 5 minutes at 2000 RPM, PBS washed and centrifuged, and resuspended in media (KGM-2 + 0.05 mM $Ca^{2+}$) containing 5% cell dissociation buffer (Gibco). The labeled CABS were first incubated with 5uM cell tracker green CMFDA in the media for 45 minutes, washed with PBS, and grown in its normal media for 30 minutes all at $37^0$C followed by digestion. The samples were analyzed by a Facscan Analyzer for cell shape.

## Characterization of CABS

*RT-PCR:*

CAB cells were media selected in KGM-2 with 0.05mM $Ca^{2+}$ and grown for RT-PCR analysis. Cells were also grown in different media as described above (table 2) for RT-PCR analysis. Cells were washed with PBS twice, and 600 µl cell lysis buffer (Invitrogen) was added to the plates. The cells were then scrapped with a cell scrapper into a 1.5 ml eppendorf tube and total RNA was isolated using a SNAP total RNA isolation kit (Invitrogen). The mRNA was reverse transcribed into cDNA which was then used in various PCR reactions to further characterize the genotype of the cell populations. PCR primers are described below in table.

AMB H 333 U 5' AGGCCAAGAGAACATGAAACT 3'
AMB H 887 L 5' CTGGAAACATGGCTCCATAG 3'

AMG 03 U 5' GGCTGCACCACCAAATCATCC 3'
AMG 04 L 5' CCCGCTTGGTCTTGTCTGTCG 3'

ENAM H 3189 U 5' GCT AGCATGACCCTTTCCTGAAG 3'
ENAM H 3710 L 5' GAATTCCTAGCCTGAAGTAGTAAGCA 3'

**Table 2.**
**PCR primers used for genotyping CAB cells**
Ameloblastin (AMB), amelogenin (AMG), enamelin (ENAM).

Positive PCR products from the ameloblastin and amelogenin specific primer reactions were cloned into a vector using a TOPO cloning kit (Invitrogen, LaJolla, California), and a positive colony picked for growth and plasmid purification using a Wizard PLUS plasmid purification kit (Promega). The plasmid was then sent for sequence analysis

(Elim Biopharamceuticals, Hayward, California). This experimental approach was described in more detail in chapter 2.

*IHC:*

CAB cells were media selected and grown on Primaria tissue culture plates and passed into 8 chambered slides (Falcon). The slides were pre-incubated with 10ug/ml of collagen type 1 (Vitrogen 50, Cohesion, Palo Alto, USA) at $37^0$C for 1-3 hour before passing the cells (to increase cell/dish adhesion), the excess collagen was then removed and the dishes allowed to dry. The cells were then grown for 1-5 days and fixed in 1% paraformaldehyde + 0.1% NP-40 detergent, washed with PBS three times and incubated with primary antibody (rabbit anti-recombinant human amelogenin, and rabbit anti-bovine ameloblastin peptide, table 3) for 1 hour at room temperature. The cells were again washed with PBS three times and incubated with secondary antibody (fluorescent labeled goat anti-rabbit) for 30 minutes at room temperature. After three more washes the cells were then analyzed by fluorescent light microscopy.

For IHC analysis of the cells stained with the biotinylated labeled secondary antibody. Primary cells were grown in KGM-2 + 0.05 mM $Ca^{2+}$ calcium, at approximately 70% confluence the media was changed to 0.05, 0.15, or 1 mM $Ca^{2+}$. After three hours the cells were fixed in 1% paraformaldehyde + 0.1% NP-40, and washed with PBS three times. The cells were then blocked in horse serum ((15 ul in 1 ml PBS), excess serum removed, and incubated with primary antibody for 30 minutes at $37^9$ C. (1:100 dilution of mouse anti-e-cadherin unpurified serum, table 3). The cells were washed in PBS once for 5 minutes. Diluted biotinylated secondary antibody (Universal) in PBS was then added

to the cells for 30 minutes at $37^9$ C (Universal Kit, Vector Laboratories). The cells were then washed in PBS one time for 5 minutes. Vectastain Elite ABC reagent was made and allowed to sit for 30 minutes (Vectastain Kit, Vector Laboratories), then added to the cells for 30 minutes. The cells were then washed in PBS one time for 5 minutes. The cells were then stained with a peroxidase substrate solution containing DAB for approximately 5 minutes (Vectastain kit). The cells were analyzed by light microscopy.

| Antibody | Working dilution | Made in | |
|---|---|---|---|
| Anti-amelogenin | 1:1000 | rabbit | |
| Anti-ameloblastin | 1:100 | rabbit | |
| Anti-e-cadherin | 1:100 | mouse | |

**Table 3.**
**Primary antibody and concentrations used in IHC of CAB cells.**

*SDS-PAGE:*

CAB cells were digested, centrifuged, washed with PBS and boiled for 10 minutes in SDS loading buffer. The cell lysate was then centrifuged and the supernatant (containing approximately 30 ug of protein) loaded on a 12% polyacrylamide gel, and separated using a constant voltage (200 volts) for approximately 50 minutes. The gel was then stained with Comassie blue for analysis or transferred to a nitrocellulose membrane for western blotting.

*Western:*

Proteins separated as described above were transferred from the 0.75mm SDS polyacrylamide gel was transferred to a nitrocellulose membrane for 1 hour 15 minutes at room temperature in transfer buffer at a constant current of 180. The membrane was then

stained with Ponceau S (0.5%) and 1% acetic acid to evaluate transfer efficiency and then blocked overnight in 2.5% nonfat dried milk and 0.1% tween in TBS. The membrane was then washed 3 times in TBS for 5-10 minutes and incubated with primary antibody at room temperature on a rocker for 2 hours. The membrane was then washed three times for 5 minutes each in TBS and incubated with secondary antibody labeled with alkaline phosphatase for 2 hours at room temperature. The membrane was again washed in TBS and stained with Sigma Fast BCIP/NBT (Sigma, St. Loius, MO, USA).

**Calcium effects on cell proliferation**

Adhesion assay:

An adhesion assay was done to determine the best method of enhancing cell adhesion after passing primary cells. First a pap pen (Zymed, California) was used to create three circles on two glass slides. These slides were then coated with 100ul 0.01% poly-L-lysine (Sigma) in the first circle, 100ul 10ug/ml type 1 collagen in the middle circle, and PBS in the last circle. The coated slides were allowed to incubate for 3 hours at $37^0$C and the excess substrate removed. Two 60mm dishes of CABS were grown in KGM-2 + .05 mM $Ca^{2+}$ media. Four hours before passing the cells the media was changed keeping it the same in one dish and the other was changed to KGM-2 + 0.15 mM $Ca^{2+}$. The cells were then passed as stated before and 10 ul of cells from a 3ml resuspension was added into each circle. The cells were examined after 24 hours for plating efficiency under an inverted light microscope. Plating efficiency was established as negligible (-), less than 50% attachment (+), 50-75% attachment (++), or greater than 75% attachment (+++).

*Proliferation assay:*

Primary CAB cells were media selected in KGM-2 with 0.05 mM $Ca^{2+}$ and grown on a 100mm dish. The cells were digested in STV for 5-10 minutes at 37°C, centrifuged at 2000 RPM for 5 minutes, washed with PBS, and spun again for 5 minutes. The cells were then reconstituted in KGM-2 media with 0.05mM $Ca^{2+}$ and counted using a hemocytometer. Before plating black 96 well plates (clear bottom) were first prepared by adding 50 ul/well of type 1 collagen (10ug/ml) for 1 hour at 37°C. The cells were then plated in a black 96 well plate (Greiner, USA), at 4000 cells/well and incubated in a humidified atmosphere at 37°C for 24 hours. (Many different cell concentrations were plated in the wells to first determine experimental conditions within the parameters of the assay that kept the cells in linear growth, this experiment established the above cell concentration of 4000 cells/well).

A cell proliferation ELISA, BrdU (chemiluminescence) kit (Roche, USA) was used to examine the proliferation rate of the cells under different experimental conditions. Briefly, after plating the cells (24hrs), the experimental media was added to the cells at a concentration of 100 ul/well. The cells were further cultured in experimental media for 24 hours. The cells were then labeled with 10 ul/well BrdU added into the experimental media (10uM final concentration BrdU), and incubated at 37°C for 2 hours. The labeling medium was removed, and the cells were fixed and denatured by addition of 200 ul/well FixDenat solution (Roche, USA), and the cells incubated for 30 min. at RT. The solution was removed and anti-BrdU-POD working solution was added to the wells (100 ul/well)

and the cells were incubated for 90 minutes at RT. The working solution was dumped and the cells were washed three times with 200ul/well washing solution. Substrate solution 100ul/well was then added to cells, the plate shaken on a rocking platform for 3 minutes and read on a fluorometer (Spectramax, Geminixs Molecular Devices corporation, California, USA) at emission wavelength 425. This experiment was repeated in triplicate with 12-18 wells/ experimental condition. A one-way analysis of variance was used for statistical analysis.

**Results:**

The human tooth germ derived primary cells were cultured in many different media conditions to further optimize and characterize these unique cells. The different media led to some unique findings with each condition. Many of these conditions involved a calcium concentration affect on the CAB cells. The characteristics of these cells in the various media are described below.

***Media selection***

*LHC8e media*

The human cells grown in LHC8e and LHC8e (10%S) produced similar results from those found in the porcine cells (Chapter 5, [10]. Cells grown in LHC8e were selective for epithelial cells, however, they were very slow growing and difficult to pass (figure 1). Passage could be performed if the cells were passed at a very high density, before the cells reached confluence. The cells could be passed 2-4 times, however, passage of the

cells was difficult and the cells would become large and vacuolated, and stop proliferation.

While the cells grown in LHC8e (10% FCS) would proliferate rapidly, this media was not selective for epithelial cells alone. These mixed culture conditions could grow past confluence. After the cells reached confluence, and sometimes prior to full confluence a cell layer would form on the basal epithelial cell layer (figure 2). When the confluent culture dishes were immunostained with an antibody against a 25 -kDa fragment of bovine amelogenin, only sparse spot like reactions were seen (figure 2).

*Cells grown in KGM-2 with serum*

The cells grown in KGM-2 with different concentrations of serum (10%, 5%, and 2%) and 0.05mM all produced similar results when analyzed by light microsopy. The cells cultures were of mixed origin and initially contained epithelial clones and fibroblast-like spindle shaped cells scattered throughout the dish. As the cells reached confluence the spindle shaped cells surrounded the epithelial pockets. Initially the spindle shaped cells surrounded encircled the edge of epithelial colonies with a few cell layers thick tangential to the round epithelial colonies, walling off these cells. The spindle shaped cells would then continue proliferation, but these cells would change direction, with the length of the cell perpendicular to the epithelial colonies (figure 3). These cells could grow for months and did not die at confluence, rather over time the epithelial cells seemed to show elongated projections on top of the clones. These projections were later shown through

TEM analysis to be mesodermal cells growing on top of the epithelial cells (figure 4). The epithelial cells grown in serum conditions seemed to have increased granularity in their cytoplasm. The serum conditions did select for spindle shaped cells (SPABS), which are further characterized and reported in the following chapter (Chapter 6).

*Cells grown in KGM-2 with different calcium concentrations*

Cells grown in serum free KGM-2 with different calcium concentrations exhibited many differences. The cells grown in a 0.05 mM calcium concentration revealed an initial mixed culture, with epithelial colonies, and sparse spindle like cells. Without the presence of serum, the epithelial cells took over the spindle shaped cells producing a primary cobble stoned shaped epithelial human tooth germ derived cell population that I refer to as CABS (cobble stone shaped ameloblast like cells). The cells selected in KGM-2 + 0.05 mM calcium proved to have the most selective advantage for CAB cells. These cells showed no intercellular bridging when observed under light microscopy (figure 7). TEM revealed tonofibrils concentrated in the perinuclear region of the cytoplasm, and no typical attachment apparatus (figure 5).

Cells grown in serum free KGM-2 with 0.15 and 0.25 mM calcium concentrations gave similar results. The initial conditions showed more spindle type cells forming as compared to 0.05mM calcium, however the pattern of epithelial clone formation surrounded by the spindle-shaped cells was similar in initial culture conditions with 0.05, .075, 0.15, and 0.25 mM calcium (figure 6). Media with 0.25 mM calcium supported

more spindle shaped cells than media with 0.15 mM calcium, which supported more spindle shaped cells than 0.05 mM calcium overtime.

The cells grown at calcium concentrations above 0.05 mM showed intercellular bridge formation, this intercellular bridge formation would occur within 1 hour after the addition of increased calcium (figure7, 8, 9, and 10). The epithelial cells in 0.25 or 0.15 mM calcium seemed to proliferate faster, than the cells grown in the same media with 0.05 or 1.0 mM calcium. Further analysis of cell proliferation in different calcium concentrations is described below.

Cells were also grown in serum free conditions with 1.0 mM calcium concentration. When cells were initially plated at these conditions, an increased background of spindle type cells was observed compared to cells at lower calcium concentrations (figure 11). These initial conditions supported the growth of epithelial cells, however epithelial growth did not spread in a monolayer, as observed in the cells grown at 0.05mM calcium, rather the cells would grow upward, piling on top of each other (figure 11 and 12). This nodule formation was observed in the middle of the epithelial colony, and was analyzed by light and transmission electron microscopy (figure 12). Immunostaining with the 25-kDa anti-amelogenin antibody revealed positive staining when observed by TEM (figure 12 and 13). Nodule formation would also occur when epithelial cells were initially selected for in KGM-2 + .05 mM $Ca^{2+}$, and then transferred to the same media with 1.0 mM calcium. These cells also showed intercellular bridge formation within 1 hour of increased calcium concentration (figure 7). The intercellular bridge formation was

reversible by returning the cells to a lower calcium (0.05 mM) media. The cells that were imaged by TEM after the addition of 1.0 mM calcium from KGM-2 + .05 mM $Ca^{2+}$ revealed desmosme like structures with attached plaque structures that were associated with tonofibrils (figure 10).

***Characterization of CABS grown in KGM-2 with 0.05 mM calcium:***

These studies showed that the most reliable method to select for CAB cells was using KGM-2 + 0.05 mM calcium. Further characterization of the CABS, selected in low calcium showed protein expression characteristic for ameloblasts.

*RT-PCR:*

PCR performed on cDNA that had been reverse transcribed from isolated cellular RNA in primary CABS revealed positive tooth specific gene PCR products. After conformation, three primer pairs each specific to a separate gene sequence of ameloblastin, amelogenin, and enamelin were used to analyze primary CAB cells. The ameloblastin specific PCR primers generated a positive PCR product of 554 base pairs in CABS as well as from cDNA using the human tooth germ library controls. The amelogenin specific PCR primers generated a positive PCR product of 383 base pairs using cDNA from CAB cell types, as well as the human tooth developmental library control. This same type of analysis was done using an enamelin specific PCR primer pair with a positive base pair product of 521, again using CABS and the human tooth

developmental library cDNA control. (Figure 14). The amelogenin and ameloblastin human PCR products were confirmed by DNA sequencing.

To further confirm that the tooth specific mRNA was coming from the CAB cells, SV-40 transfected CAB cells were clonally isolated. This clonal CAB cell population was then analyzed to confirm the presence of amelogenin specific to the CAB cells. (Data not shown).

*IHC:*

Immunohistochemistry using an anti-amelogenin antibody was performed on primary CAB cells grown in KGM-2 + .05 mM $Ca^{2+}$. The CAB cells revealed a positive nuclear staining pattern compared to pre-immune controls (figure 15).

CAB cells initially selected for in KGM-2 + .05 mM $Ca^{2+}$ and then media changed, using three different calcium concentrations in the media revealed different staining patterns with e-cadherin. Cells grown in KGM-2 + .05 mM $Ca^{2+}$ showed minimal staining with the e-cadherin antibody, in contrast, cells that were transferred into 0.15 or 1.0 mM calcium showed positive staining for e-cadherin intercellularly, with an increased staining in media containing 1.0 mM calcium. (Figure 16).

*Western:*

Western blot analysis on primary CAB cells revealed a positive staining pattern for two tooth specific proteins (ameloblastin and amelogenin). A positive control of tissue that was digested, according to the cell culture protocol, but never plated also stained with antibodies against ameloblastin and amelogenin. The anti-ameloblastin antibody used in Western staining revealed three distinct bands at 50, 52 and 62 kDa (figure 17). The positive control revealed a similar staining pattern, however sometimes lower bands at 48 and 46 kDa were also seen. The anti-amelogenin antibody revealed the main lower molecular amelogenin band at 25 kDa in the positive control with higher molecular weight aggregates, however only higher molecular weight bands of amelogenin were seen in the CAB cells (figure 18).

*Cell adhesion*

Cell adherence was increased almost two fold with the addition of collagen type 1 onto a glass slide. Poly-L-lysine proved not to be effective to increase cell adhesion of the epithelial primary cells. PBS also did not promote adhesion of the epithelial cells. Collagen type 1 was used to coat the plates for cell adhesion during the proliferation assay.

*Proliferation*

A growth curve was established on the primary CAB cells to determine cell concentrations that produced linear growth within my experimental parameters (figure 19). This was used to establish a single cell concentration at 4000 cells/well that was used to analyze the effects calcium had on human epithelial tooth germ cell proliferation.

There was a dose dependent response with calcium that reached a threshold and then reversed its effect. Cells grown in KGM-2 + 0.15 mM calcium had the fastest rate of proliferation followed by media with 1mM calcium, then media with 2% FCS and 0.05 mM calcium, followed by media without serum and 0.05 mM calcium (figure 20). The increased rate of proliferation was statistically significant in comparing media with 0.15 mM calcium to, KGM-2 media containing 0.05 ($P<0.001$), 1.0 mM ($P<0.001$) calcium or 2% FCS with 0.05 mM calcium ($P<0.05$). The rate of proliferation was also statistically different between media with 1.0 mM calcium and media with .05 mM calcium ($P<0.001$), and media containing 0.05 mM calcium versus the same media with 2% FCS ($P<0.05$). The proliferation rate between media with 1.0 mM calcium and 0.05 mM calcium with 2% FCS was not statistically significant.

***Shape selection of tooth germ derived cells***

*SV-40 transfection and cloning of epithelial cells:*

The CAB selected epithelial cells that were transfected with SV-40 were much more robust especially upon passage, with an enhanced rate of proliferation. They formed intercellular bridges when low calcium media (0.05 mM) was replaced with higher calcium (0.15 mM and above). Six separate epithelial colonies were isolated using the cloning ring. Each colony was expanded for further analysis. Although all 6 colonies were positive for amelogenin cDNA by RT-PCR (data not shown), only one colony was used in further studies.

*FACS analysis:*

FACS revealed that the mixed cell population could not be separated based on cell sorting by shape. A mixed cell population was grown in KGM-2 + 10%S + 0.05 mM $Ca^{2+}$, when analyzed by FACS only one population of cells was seen based on forward and sidescatter. (Figure 21).

FACS was also done on individual cell populations. The FACS analysis revealed that SPAB cells showed a similar forward and side scatter pattern to the mixed cell populations. When the SV-40 clonally isolated epithelial cell population (CABS) were fluorescently labeled and analyzed for fluorescence (CMFDA) and forward scatter, a labeled cell population was shown. These same CAB cells, however, revealed a similar forward and side scatter to the SPAB cells and the mixed cell population. When the labeled CAB cells were mixed with unlabeled SPAB cells and measured by fluorescence (CMFDA) and forward scatter, two distinct cell populations were shown. In contrast, measuring the labeled (CABS) mixed with the unlabeled SPABS in forward and side scatter showed similar results to those grown in mixed cell culture. (Figure 22).

**Discussion:**

This is the first study to report the characterization and growth of human primary tooth germ derived cultures. The initial culture conditions, derived from earlier studies (Chapter 5, [10, 12], when applied to human tooth germs produced similar results. These conditions allowed the growth of epithelial colonies, however, they also produced a large background of other non-epithelial looking cells. All previous studies of primary cells derived from tooth germs have reported a mixed cell culture [6, 8-10]. This report is

unique, not only in the culture of human tooth germs, but the ability to consistently grow non-mixed primary epithelial cultures.

Different calcium concentrations were utilized in serum free media to select epithelial cells from primary tooth germ cultures. The keratinocyte growth media supplemented with low calcium, (KGM-2 + 0.05 mM $Ca^{2+}$) proved most useful in the establishment of a primary CAB cell population. These cells showed a phenotype characteristic for epithelial cells.

Previous studies by Kukita and coworkers described the primary culture of tooth germs (from rat incisors) in serum free media [9] utilizing 0.1mM calcium. Their results showed a highly mixed culture. In our laboratory LHC-9 (which contains 0.11mM calcium) was used to grow primary porcine tooth germ cells in serum free conditions, however, a similar mixed culture to those found by Kukita and coworkers was grown in this media [10]. My studies showed a mixed culture in different media at a similar calcium concentration (KGM-2 + 0.15 mM calcium). However at lower calcium concentrations I successfully selected a cell population with a classic appearance of epithelial cells.

Although, the culture conditions in low calcium proved successful for the primary culture of CABS, my initial cultures were very difficult to consistently pass. Kukita and coworkers overcame the cell passage problem in both primary and secondary tooth germ derived cell cultures with the use of collagen coated plates [9]. I found collagen type I coatings (Vitrogen, Cohesion, Palo Alto, Ca) increased the adhesion of the CAB cells,

which was useful for IHC and proliferations assays. Our lab utilized Primaria (Falcon) dishes carrying a positively charged surface [10], which was very successful for initial plating, however, I found was not consistently successful for further passages.

The literature reports conflicting evidence for different cell types producing enamel proteins. Initially it was reported that the enamel organ epithelial cells produced amelogenin, analyzed by Western blotting [6]. Later reports by Kukita and coworkers confirmed amelogenin expression, not in the epithelial cells, but in spindle-shaped cells [9]. To reconcile these conflicting reports I set out to isolate a colony of the cobble stoned shaped epithelial like cells from all other cell types.

The CAB cells selected in KGM-2 with 0.05 mM calcium expressed enamel matrix proteins. To confirm their genotype I transfected these cells with SV-40, which allowed me to expand the cells and characterize the transfected cells derived from a single colony. The primary CAB cells grown in KGM-2 + 0.05 mM calcium were difficult to pass. The problem of cell passage was overcome by SV-40 transfection of human tooth germ derived epithelial selected cells. After transfection these cells greatly increased their rate of proliferation, and would adhere and establish colonization of a dish at very low densities. The human SV-40 transfected cells allowed me to isolate, pass and expand cells from a singly colony for biochemical analysis. These cells were further analyzed by RT-PCR exhibiting positive results for amelogenin. This analysis was especially important in these early stages of characterization to confirm the presence of tooth

derived messages (amelogenin) from a single epithelial colony. This allowed me to confirm that the epithelial cell type produced tooth specific proteins.

The specific origin of the epithelial cell type is not known. It is possible that the CAB cells are derived from a specific stage of epithelial ameloblasts, which would explain the protein expression of these cultured cells. However other epithelial derived cells are also present in tooth germs such as stratum intermedium and stellate reticulum. Kukita and coworkers hypothesized that the cobble stone shaped epithelial cells differentiated into the spindle shaped cells, and differentiation from early to later stage ameloblasts occurred [9]. In primary culture the addition of serum into the CAB selection media did lead to increased spindle shaped cell formation, though this is likely due to upregulation of these spindle shaped cells, and not differentiation. If primary CAB cell populations were grown in KGM-2 + 0.05 mM calcium the spindle shaped cells could be selected against over time, where additional spindle shaped cells did not grow even in the presence of serum. Further studies were warranted to characterize the spindle shaped cells. The spindle shaped cell characterization is presented in chapter 6.

Shape selection studies were also performed to isolate non-transfected cells (CABS and spindle shaped cells). A fluorescent activated cell sorter (FACS) was used to isolate individual cell populations based on shape and granularity. These initial experiments were designed to separate the predominant cell culture populations through FACS, which at the time was difficult to do based on media selection alone. Mixed cultures have similar forward and side scatter characteristics, which was unexpected due to the

different characteristics of the epithelial and spindle-like cells observed during culture. I confirmed this observation by fluorescently labeling an isolated cell population of SV-40 transfected CABS and mixing them with unlabeled spindle shaped cells.

These SV-40 transfected CAB labeled cells were then analyzed alone and mixed together with an unlabeled spindle shaped cell population. The result was confirmed that two cell populations (epithelial and spindle shaped cells) exist with the same forward and side scatter characteristics, within a mixed cell population. When a fluorescently labeled epithelial cell population was mixed with an unlabeled spindle cell population, two populations were sorted through FACS analysis. However, when both the epithelial and spindle shaped cells are unlabeled, only one group of cells is observed through FACS. Although previous studies of rat incisor enamel organ using FACS analysis have selected cells that express amelogenin using an anti-amelogenin antibody, these cells were fixed and therefore could not be cultured [13]. Since FACS analysis could not be used to separate a mixed cell culture population other methods, including the reliance of the cells on calcium, were exploited.

The tooth germ derived culture experiments were affected by calcium concentration in a number of different ways. First, the amount of calcium in the KGM-2 media changed the phenotype of the cells. The relative number of spindle-type cells in the cell cultures was directly proportional to the amount of calcium in the cell culture media. Although spindle shaped cells could be seen in the initial cultures of KGM-2 + 0.05 mM calcium, they would not proliferate, but were slowly selected against in these cell culture

conditions. This reduction in spindle-shaped cells did not occur when calcium was increased to 0.075, 0.15, 0.25, or 1.0 mM with an increased support for spindle-shaped cells as the concentration of calcium was increased.

The CAB cells also behave differently depending on the media calcium concentration. General observations were that when the medium concentration was increased to 0.15 or 0.25, the cells seemed to proliferate faster than those at 0.05, or 1.0 mM calcium concentration in the same media. This general observation was confirmed, through a proliferation assay, that epithelial cells proliferated at a much greater rate when 0.15 mM calcium was in the media versus 0.05 or 1.0 mM. Mouse keratinocyte proliferation is also controlled through calcium concentration in cell culture [2].

The concentration of calcium in the media also had a profound effect on intercellular bridge formation with the epithelial cells involving e-cadherin. Immunolocalization studies confirmed the upregulation of e-cadherin with the increase of calcium in the media. Light microscopy showed that increasing the calcium concentration from 0.05 to 0.15 or 1mM lead to intercellular bridge formation. This analysis was confirmed by TEM observations. Previous studies on keratinoycte cultures have also shown intercellular bridge formation due to increased calcium [2]. Further, immunolocalization studies have localized e-cadherin to the inner and outer enamel epithelium during tooth development [14]. Further studies are necessary regarding intercellular adhesion molecules and their role in tooth development.

Immunohistochemistry was completed to further characterize the CAB selected cells. The staining pattern of amelogenin, the most abundant protein in enamel formation was used to evaluate the tooth specific nature of these cells. The amelogenin staining pattern found in CABS was unexpectedly located to the nucleus where one would expect some type of control over transcription. Amelogenin is secreted by ameloblasts into the enamel matrix [1]. Current literature supports the hypothesis that amelogenin guides the formation of organized crystal growth in enamel formation, it is not hypothesized to control transcription [15] [16]. Recently, amelogenin has been implicated in the stimulation of bone [17, 18], which may imply transcriptional control. Although amelogenin is the most studied protein, its analysis is complicated further by the presence of at least 14 splice variants [19] (personal communication, Wu Li). The function of each of these variants remains elusive. Our IHC analysis used a full-length recombinant amelogenin , which would immunostain a number of different splice variants [19, 20]. Future studies will be aimed at confirming the nuclear localization and determining whether the staining pattern found with the tooth germ derived epithelial cell is related to a novel function of amelogenin in these cells.

Confirmation of amelogenin expression by the CAB cells was done through Western blotting. The pattern of amelogenin staining was similar to the upper bands, but not the lower bands relative to the positive control. The higher molecular weights of amelogenin are likely the self-assembly of amelogenin [21], as the bands match those that would form due to aggregates of the 25 kD amelogenin subunit (50-, 75-, 100-, and 200 kDa). An earlier study reported that epithelial cells derived from tooth bud explants, when

analyzed by Western blotting using a porcine amelogenin antibody, produced positive bands of 50- and 53- kDa [6]. Further studies will be completed using pre-immune controls to confirm the positive staining of the amelogenin protein.

CAB cells also stained positive for ameloblastin when analyzed by Western blotting. The pattern of ameloblastin staining was very similar to that seen in the positive control enamel organ cells, however, as we develop new antibodies this work will be confirmed. The ability to grow "ameloblast-like" cells in serum free culture conditions that express ameloblastin will allow further characterization of this little studied enamel protein. This type of analysis will prove to be a useful tool in deciphering the complexity of tooth development and mineralization.

**Conclusion:**

In this chapter I have described studies that significantly advance our ability to selectively grow and culture cells derived from the human tooth enamel organ. Using the hypothesis, that the tooth germ epithelial cells behave similar to keratinocytes, I was able to use different calcium concentrations to selectively isolate characterize cells. These results allowed me to establish a primary human epithelial tooth germ cell culture and to analyze the effects that calcium had on this culture. Calcium regulated the cells in many different ways by selecting for a primary epithelial cell population, leading to the formation of intercellular bridging, upregulating e-cadherins, promoting nodule formation, and controlling cellular proliferation. These cells seem to produce amelogenin, and ameloblastin protein and express enamelin mRNA signals, however, as

the cells are further characterization, work confirming the presence of the enamel proteins will prove useful. These primary studies generate a human tooth derived primary epithelial cell culture will prove useful in future studies regarding tooth development and tissue engineering.

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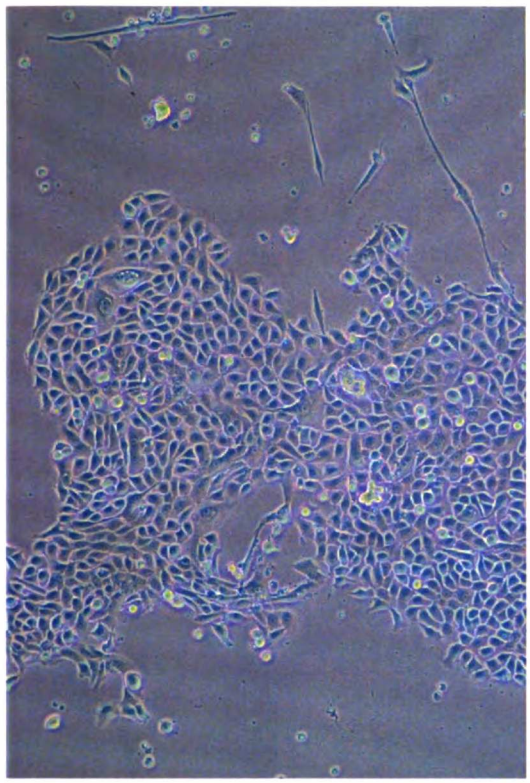


**Figure 1.**
**Phase contrast image of human enamel organ derived cells grown in LHC8e (10X).** Cobble stone shaped epithelial cells were selected for with some background of spindle shaped cells. These cells could be maintained for 3-4 weeks, and then became vacuolated as reported with porcine enamel organ derived cells (Chapter 4).

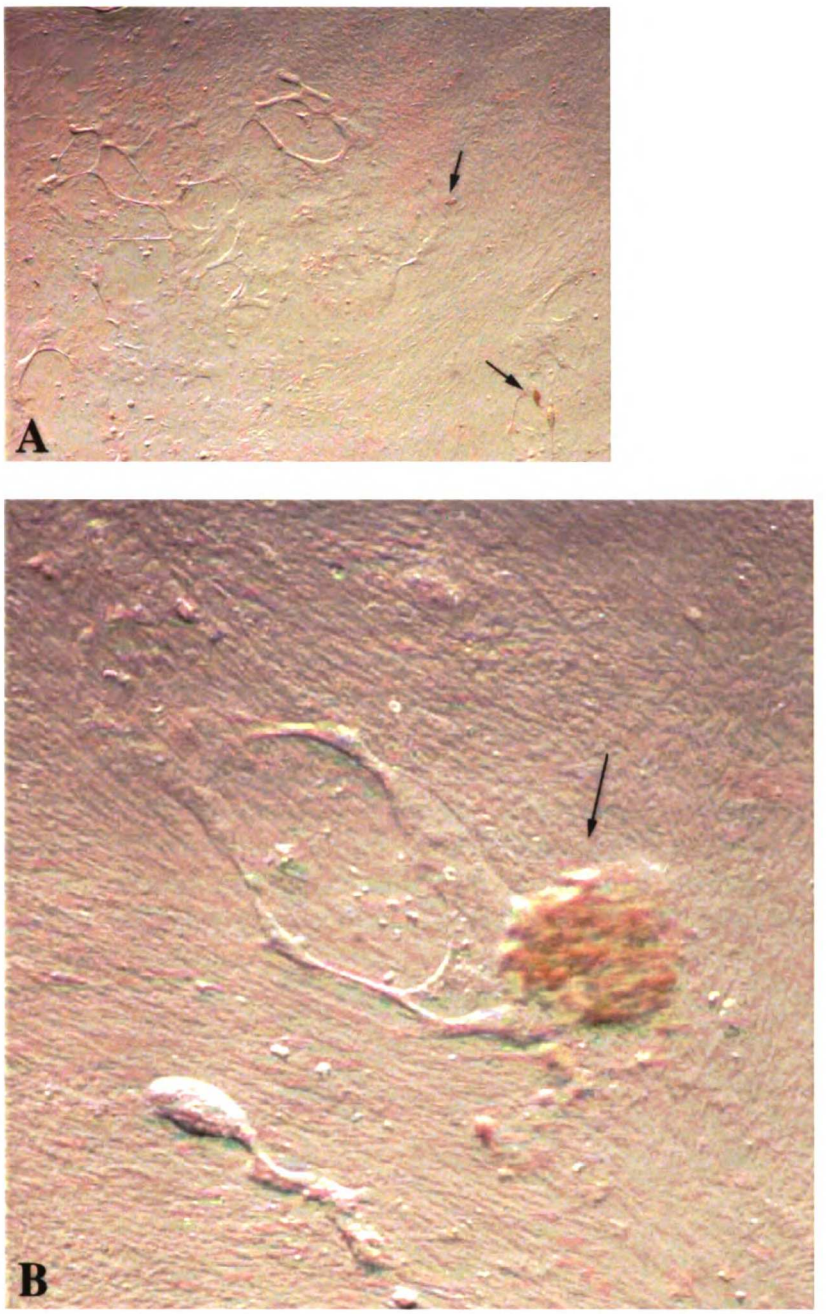



**Figure 2.**
**Phase contrast image of human cells grown in LHC8e + 10% (FCS).**
Arrows show positive spot-like immunoreactions with an anti-amelogenin antibody, however, they were seldom seen. (A) Image at 4X. (B) Image at 20X. Both images show another cell layer growing on top of the confluent basal cell layer.

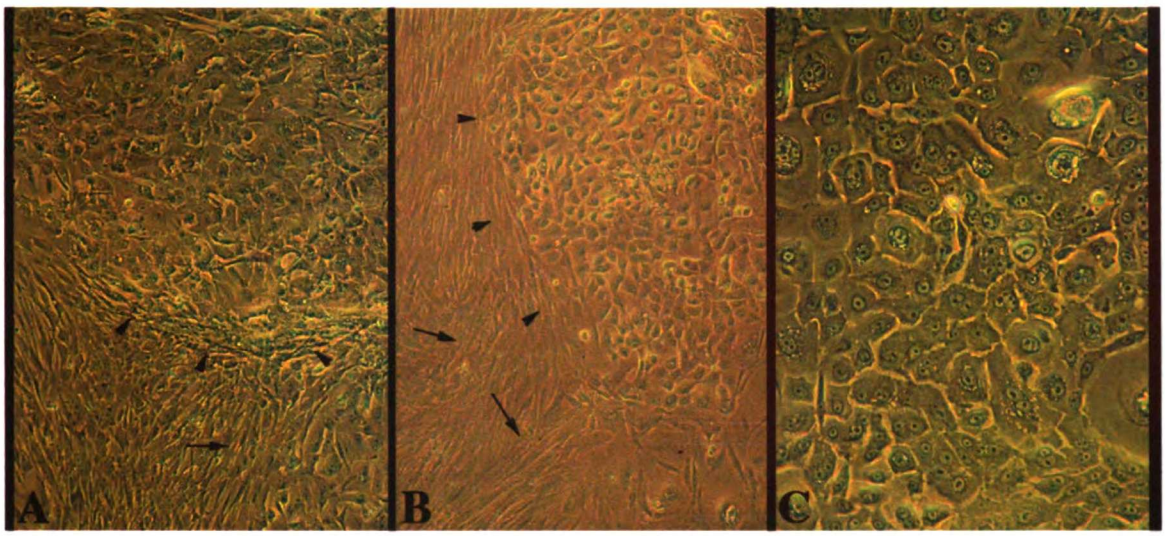



**Figure 3.**
**Phase contrast image of human enamel organ derived cells grown in KGM-2 media with different serum concentrations and 0.05mM calcium.**
(A) Cells grown in KGM-2 with 10% FCS and 0.05mM calcium show an epithelial colony surrounded by spindle-shaped cells (10X). (B) Cells grown in KGM-2 with 5% FCS and 0.05mM calcium (10X). (C) Cells grown in KGM-2 with 2% serum and 0.05mM calcium, close up of the epithelial colony shows increased granularity compared to cells grown without serum. The spindle shaped cells run perpendicular to the cobble-stone epithelial cell clones as shown by the small arrowheads in A and B. The spindle shaped cells then change direction as shown by the arrows in A and B.

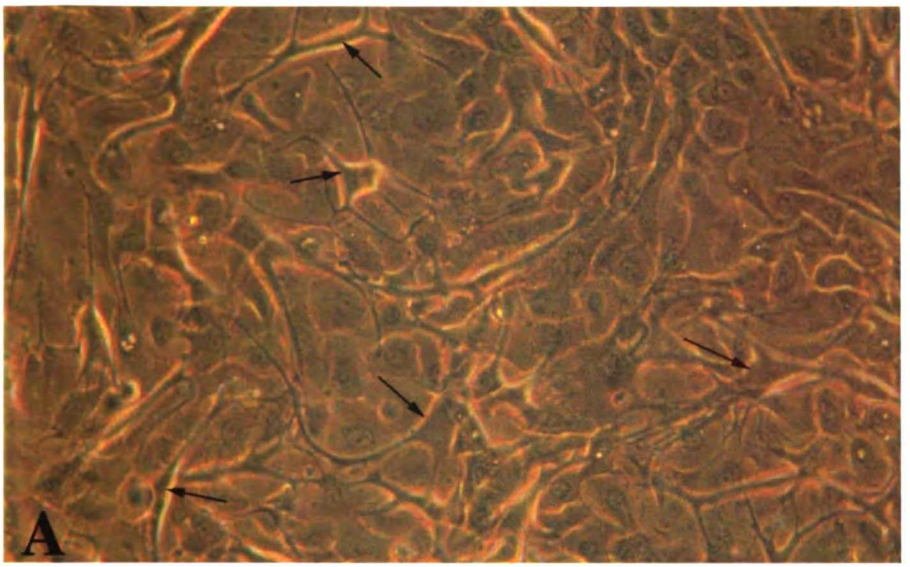



**Figure 4.**
**Phase contrast image of CAB cells grown in KGM-2 + 0.05 mM calcium (20X).**
(A) Elongated projections (arrows) appeared on the epithelial colonies if the cells were left in culture for about 4-5 weeks, however the cells could not be sustained at confluence. The elongated projections originally were thought to be extracellular matrix, however, TEM images showed the projections to be dendritic cells. CAB cells are underneath the dendritic cells.

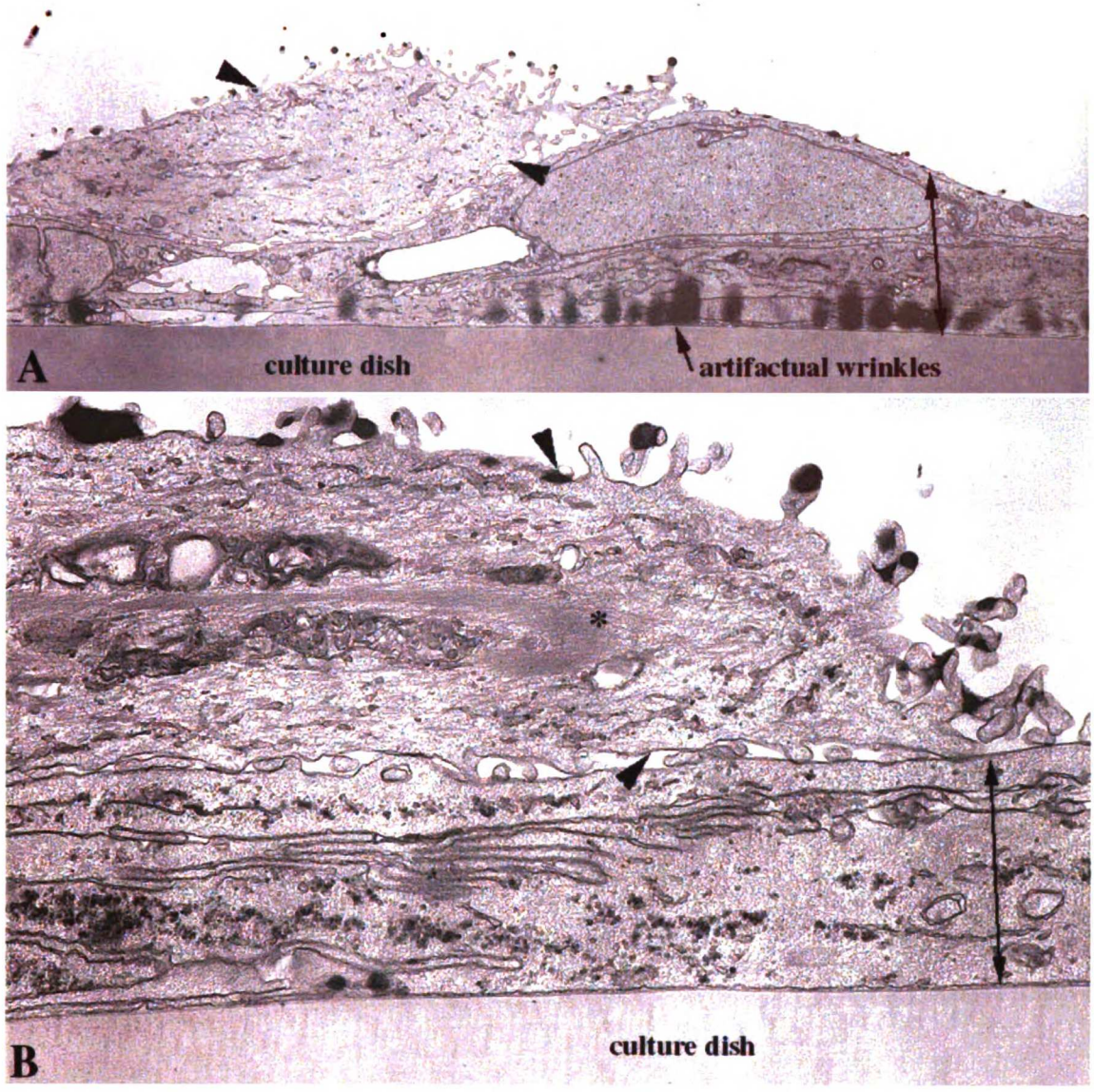



**Figure 5.**
**TEM analysis of CAB selected cells in KGM-2 + 0.05 mM calcium with dendritic cells on top of basal CAB layer.**
(A) Sectional view of CAB cell with a dendritic cell on top. (B) Same profile as A, at increased magnification. The dendritic cells contain bundles of tonofilament-like structures (*).
Arrowheads show dendritic cell border. These cells were shown in figure 4. Double arrowhead show CAB epithelial layer at the bottom of the culture dish. The dendritic cells are on top of the CAB cells. No hemidesmosomes or tonofilaments are seen in the basal epithelial layer due to the low calcium concentration in the media.

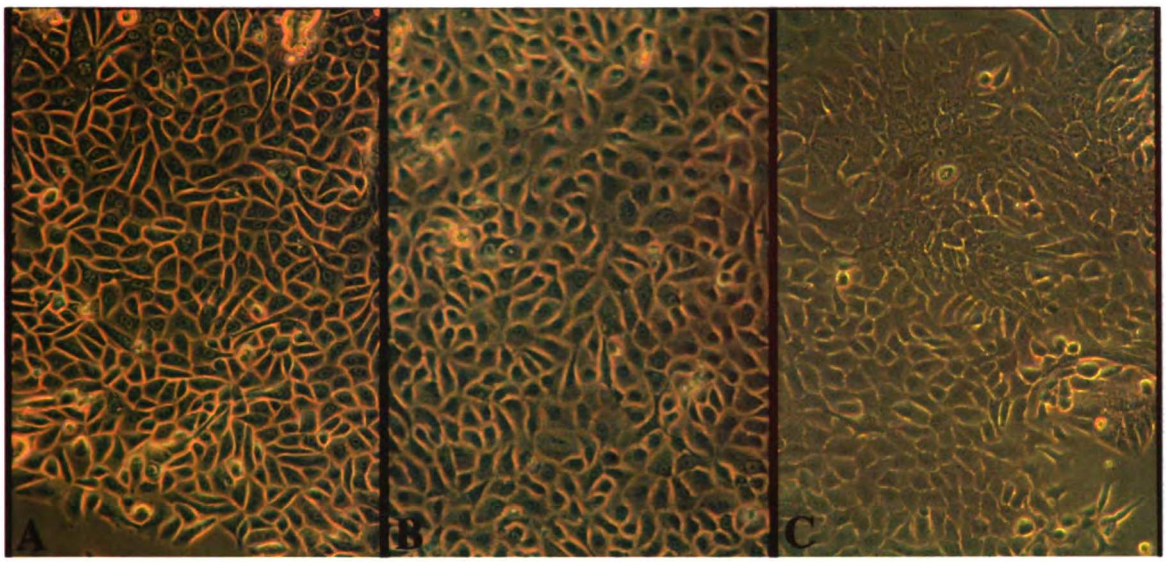



**Figure 6.**
**Human tooth germ derived CAB cells at different calcium concentrations (20X).**
(A) CAB cells selected in KGM-2 with 0.05 mM calcium (B) CAB cells selected in KGM-2 with 0.075 mM calcium (C) CAB cells selected in KGM-2 with 0.25 mM calcium. All conditions shown produced epithelial clones, however, more spindle shaped cells were seen with increasing calcium concentrations. The cells grown in KGM-2 with 0.05 mM selected almost entirely for CAB cells, therefore these conditions were used for further analysis.

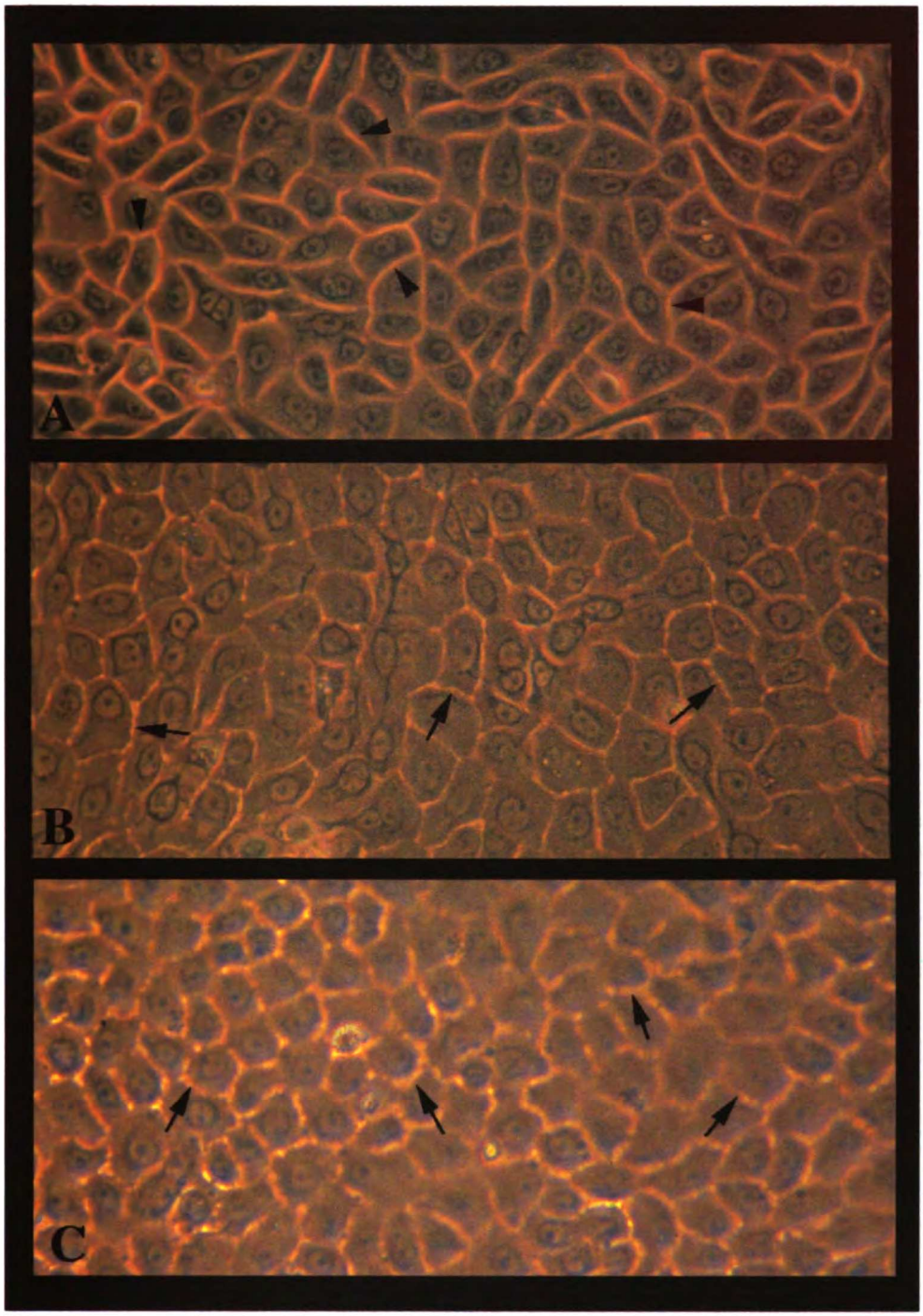



**Figure 7.**
**Phase contrast images of CAB selected cells in different calcium concentration containing media, shows intercellular bridge formation (20X).**
(A) CAB selected cells in KGM-2 + .05 mM calcium. (B) CAB cells were selected as in A, then the media was changed to contain 0.15 mM calcium for 3 hours. (C) CAB cells were selected as in A, then the media was changed to contain 1.0 mM calcium for 3 hours. Arrowheads show no intercellular bridge formation in A, arrows show intercellular bridge formation in B and C.

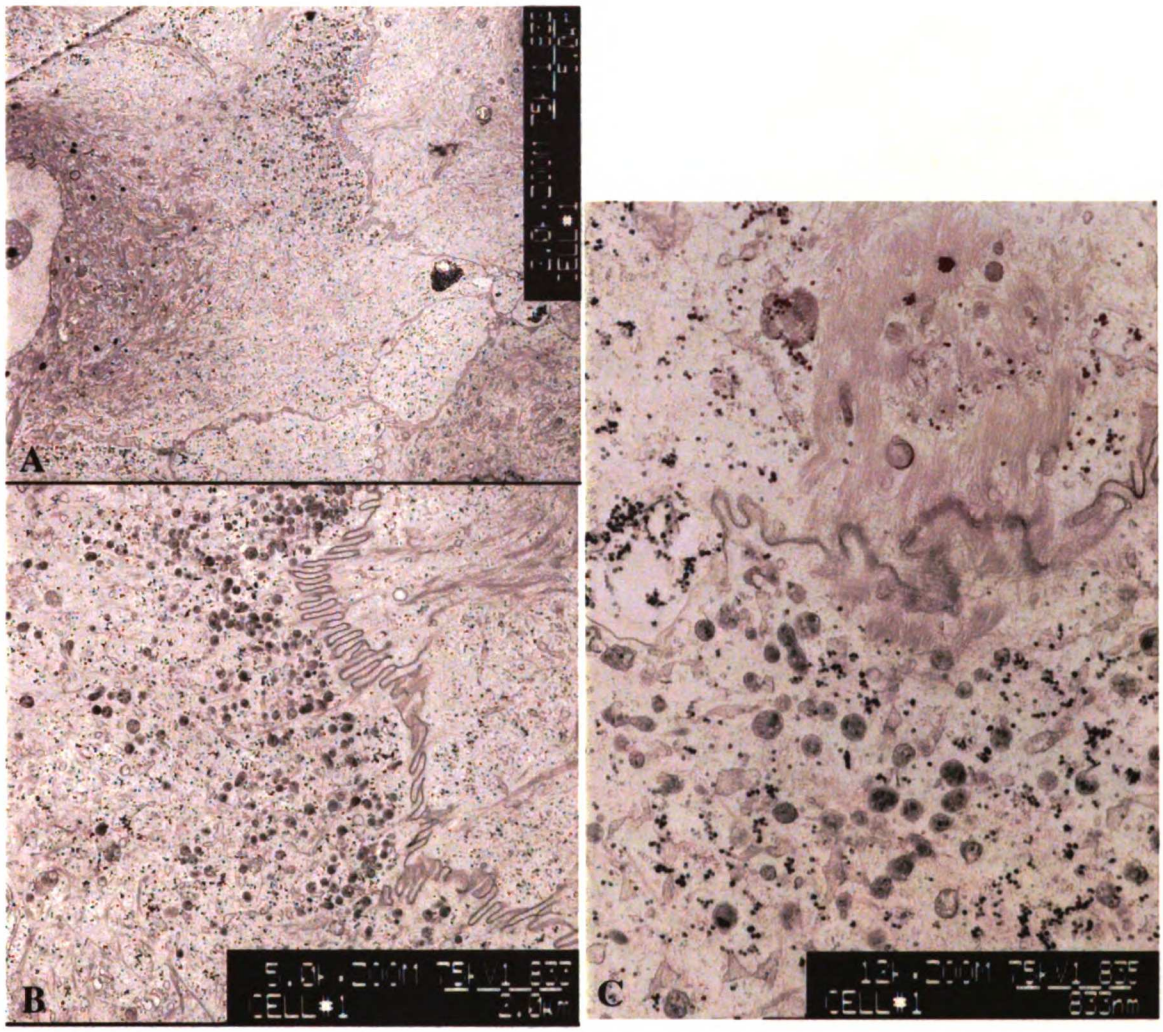



**Figure 8.**
**TEM analysis of CAB cells selected in KGM-2 with 0.05 mM calcium showing undefined intercellular bridge formation.**
(A) Tonofibrils are concentrated in the perinuclear region of the cytoplasm of most cells. No typical attachment apparatus has formed. (B) Enlargement of interdigitation where no attachment apparatus is seen. (C) Undefined attachment apparatus between cells can sometimes be observed.

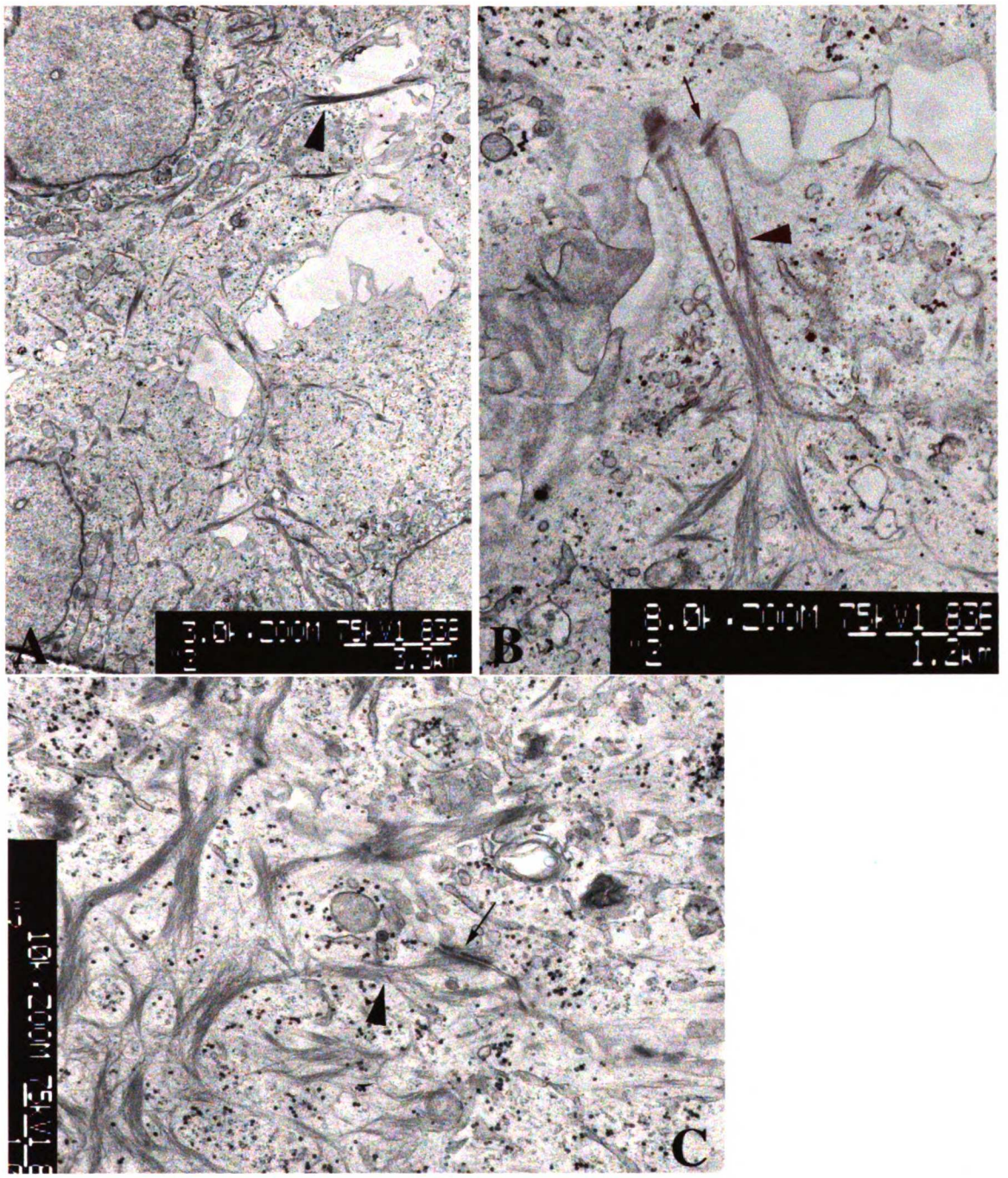



**Figure 9.**
**TEM analysis of KGM-2+ 0.05 mM calcium CAB selected cells changed to 0.15 mM calcium for 3 hours and then the cells were fixed.**
Arrowhead shows tonofibrils in A, B, and C. Small arrows show hemidesmosomes associated with the tonofibrils in B and C.

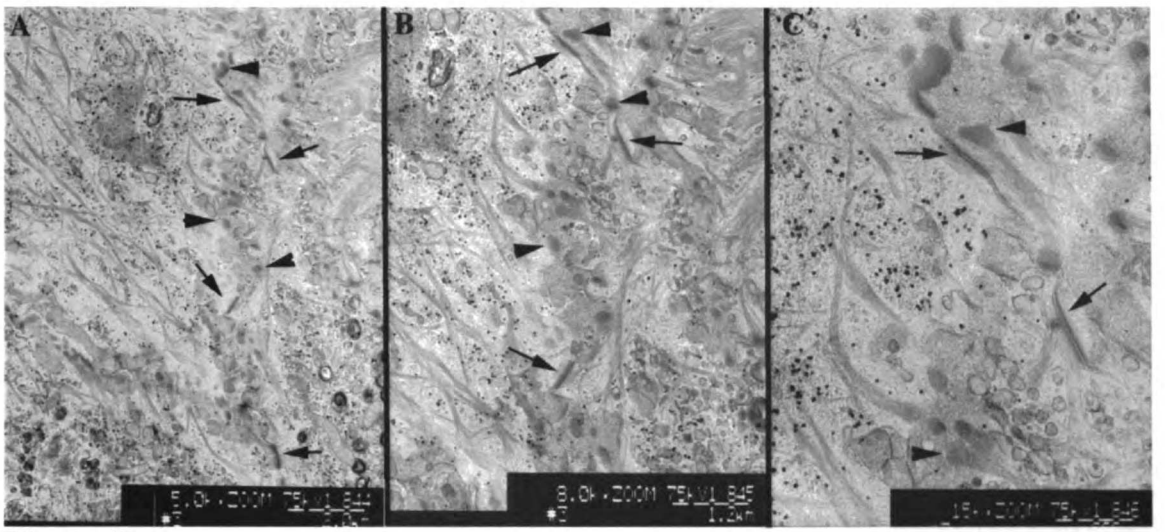



**Figure 10.**

**TEM analysis of CAB selected cells in KGM-2 + 0.05 mM calcium, then changed to media with 1.0 mM calcium for 3 hours before fixation.**

(A) Arrows show a line of desmosomes between two cells. Arrowheads show attachment of plaque-like structures. (B) Enlarged image of A reveals a closer look at desmosomes (arrows) and plaque-like structures (arrowheads). (C) Two well developed desmosomes are shown (arrows). The plaque-like structures seem to be related to the tonofibrils (arrowheads).

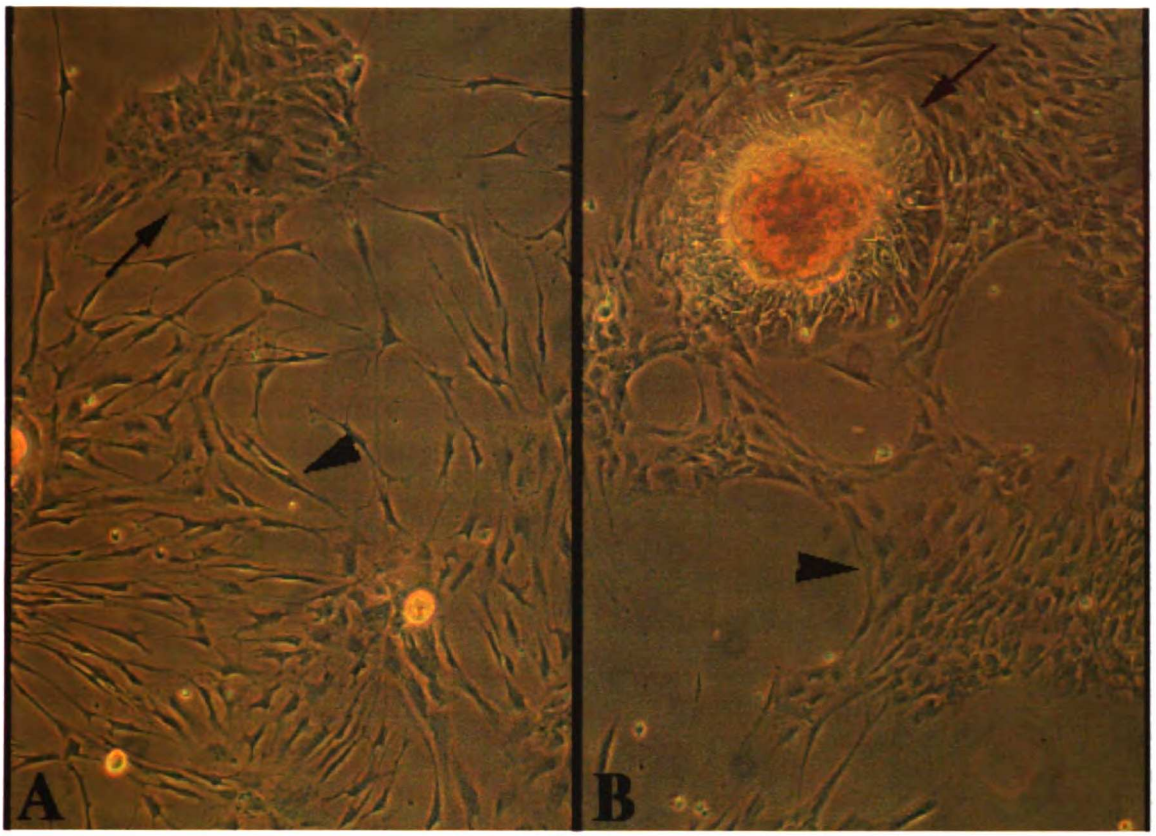



**Figure 11.**

**Phase conrast images of human derived tooth germ primary cells grown in KGM-2 + 1.0 mM calcium.**

(A) Cells were grown in this media for approximately one week. Arrow shows early formation of an epithelial colony. Arrowhead shows spindle shaped cells loosely scattered throughout the culture. (B) Cells were grown for approximately two weeks in the same media as A. Arrow shows the epithelial cells piling on top of each other, and nodule formation in the middle of the epithelial colonies. Arrowhead shows spindle shaped cells have become more adherent to each other over time.

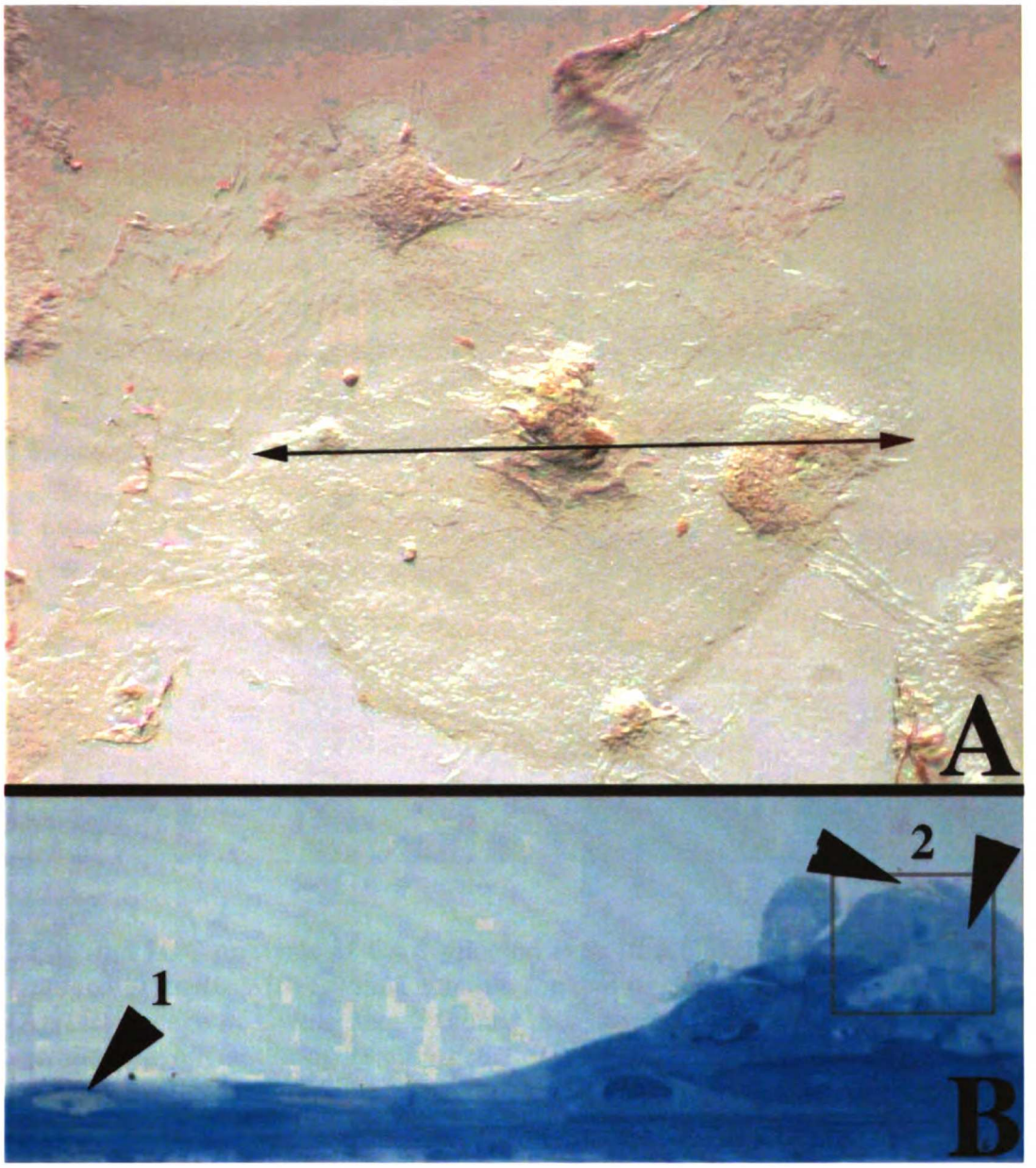



**Figure 12.**
**Light microscopy and IHC analysis of CAB selected cells in KGM-2 + 0.05 mM calcium, then changed to media with 1.0 mM calcium for two weeks.**
(A) After approximately 2 weeks in higher calcium, nodules appear in the center of the CAB colonies. Double arrow indicates where a cross section was made for further analysis. Immunoreactivity was noted in the nodule area with an anti-amelogenin antibody, (B) Light microscopy/IHC image of the nodule in cross section. Arrowheads indicate areas that stained positive with an anti-amelogenin antibody. Further analysis of positive staining was observed by TEM (figure 13).

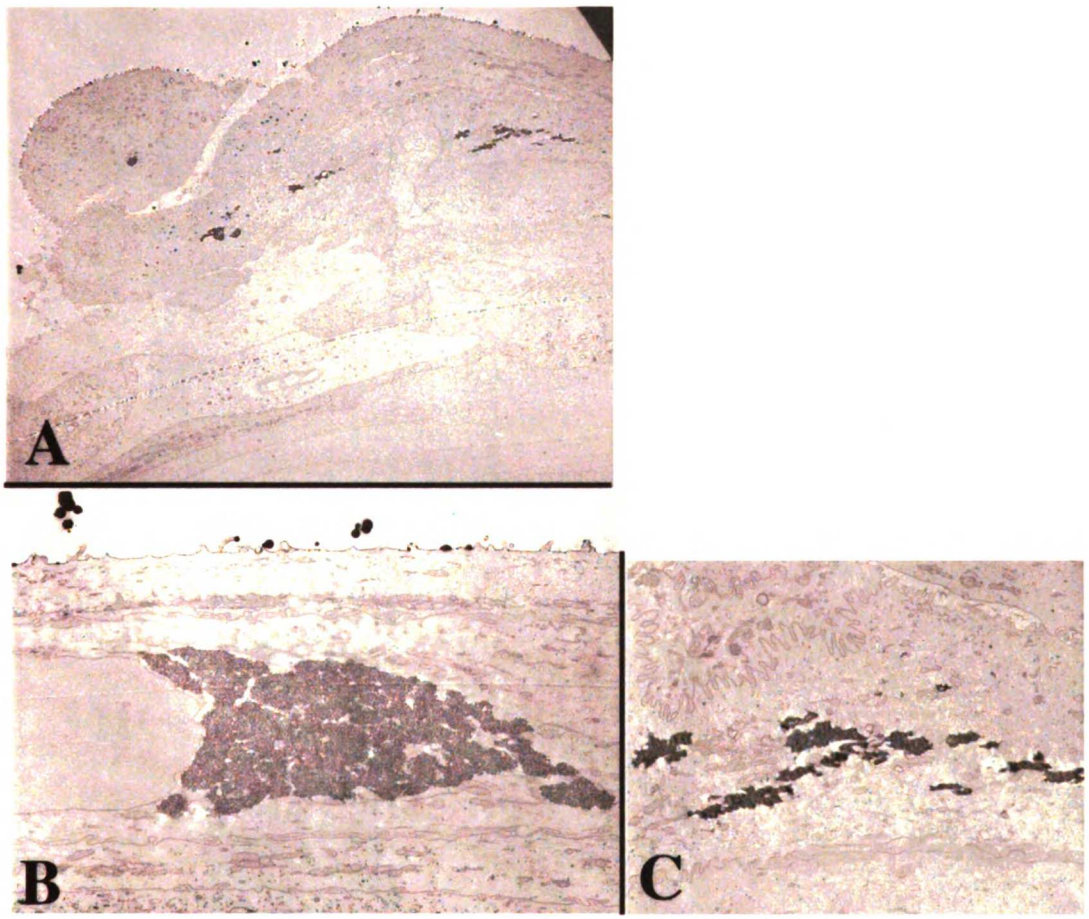



**Figure 13.**
**TEM and IHC analysis of CAB selected cells in KGM-2 + 0.05 mM calcium, then changed to media with 1.0 mM calcium for two weeks.**
(A) TEM image of nodule area 2 (boxed area) shown in figure 12. Positive reaction to anti-amelogenin antibody is enlarged. (B) TEM image of area 1 from figure 12. Immunoreaction appears to be intracellular. (C) Enlargement of area shown in A.

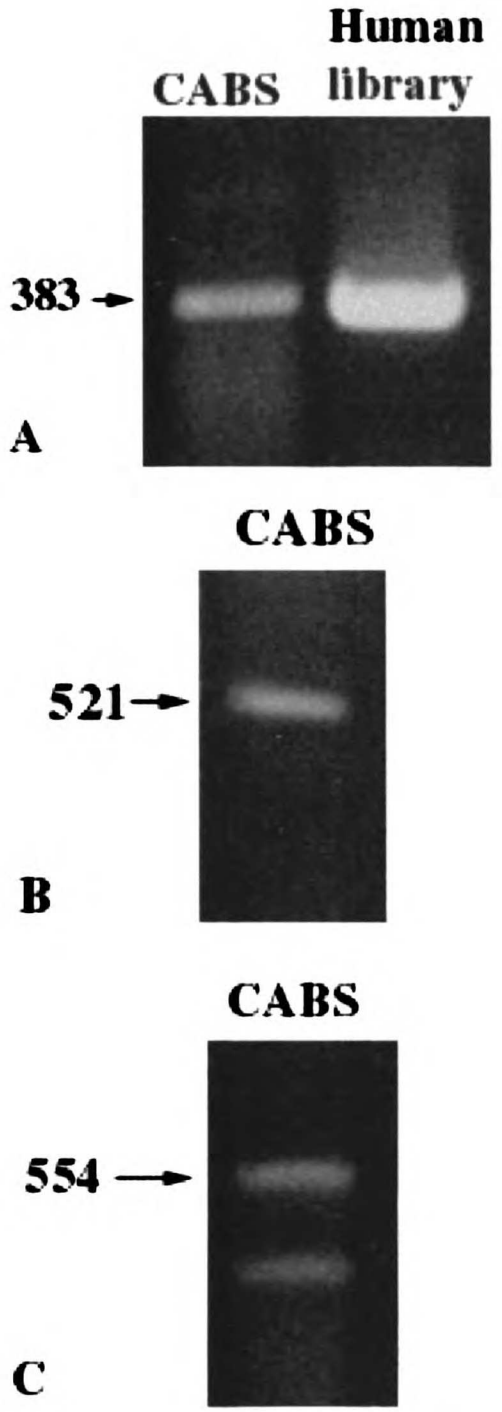



**Figure 14.**
**PCR of cDNA from CAB cells selected in KGM-2 + 0.05 mM calcium.**
(A) Amelogenin specific primers were used to generate a positive PCR product of 383 base pairs in the cell cDNA and a human tooth developmental cDNA library control. (B) Enamelin specific primers generated the correct size of PCR product (551 base pairs) using CAB cellular DNA. (C) Ameloblastin specific primers were generated the correct size of PCR product (554 base pairs) using CAB cellular cDNA.

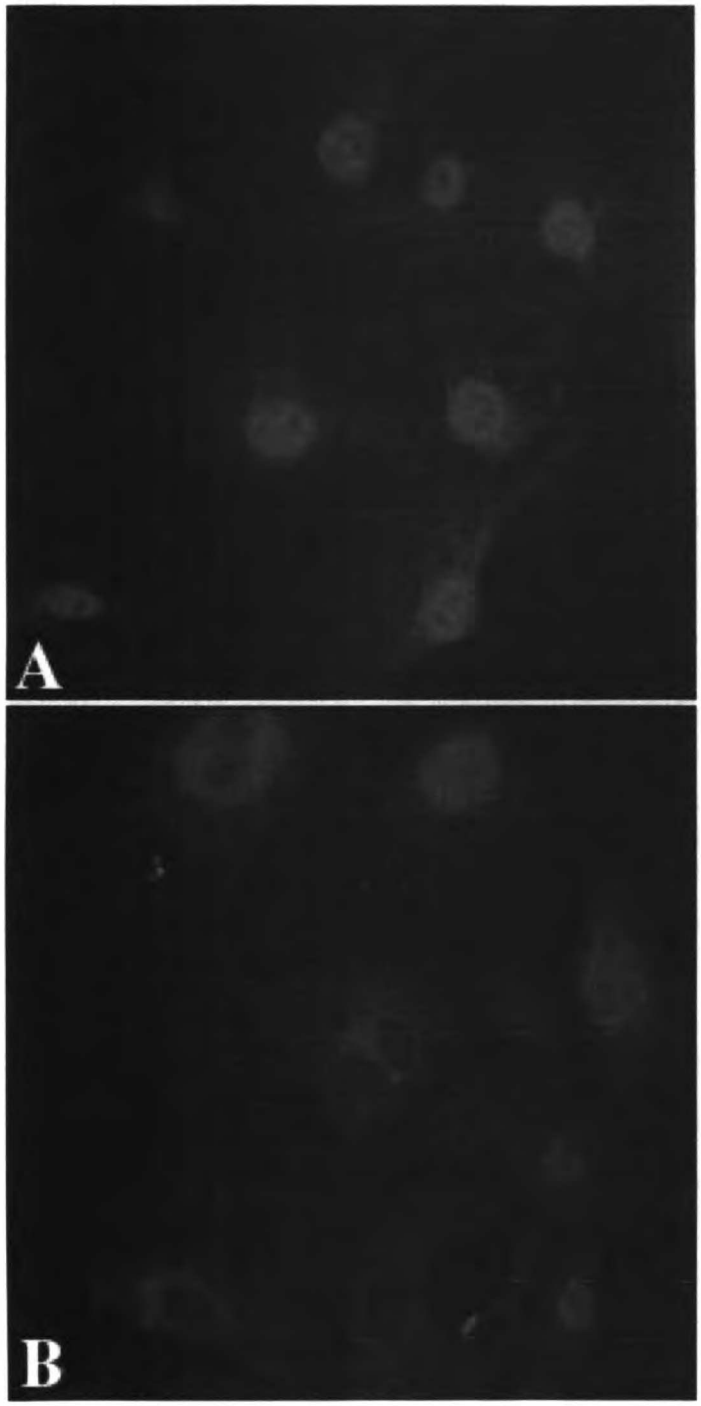



**Figure 15.**
**Fluorescent light microscopy image of CAB cells selected in KGM-2 + 0.05mM calcium.**
(A) Positive nuclear staining was observed in the CAB cells with a 1:1000 anti-recombinant amelogenin antibody. (B) Negative staining was observed with the CAB cells with a 1:1000 pre-immune serum from the same animal that the antibody was made. A 1:1000 dilution of fluorescently labeled secondary goat anti-rabbit antibody was used in A and B.

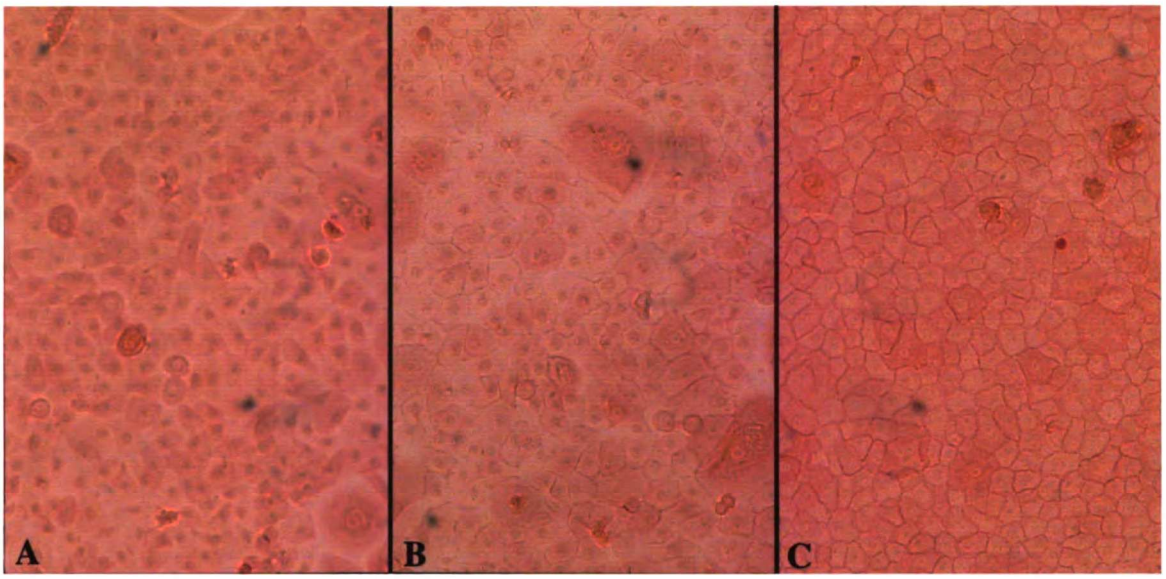
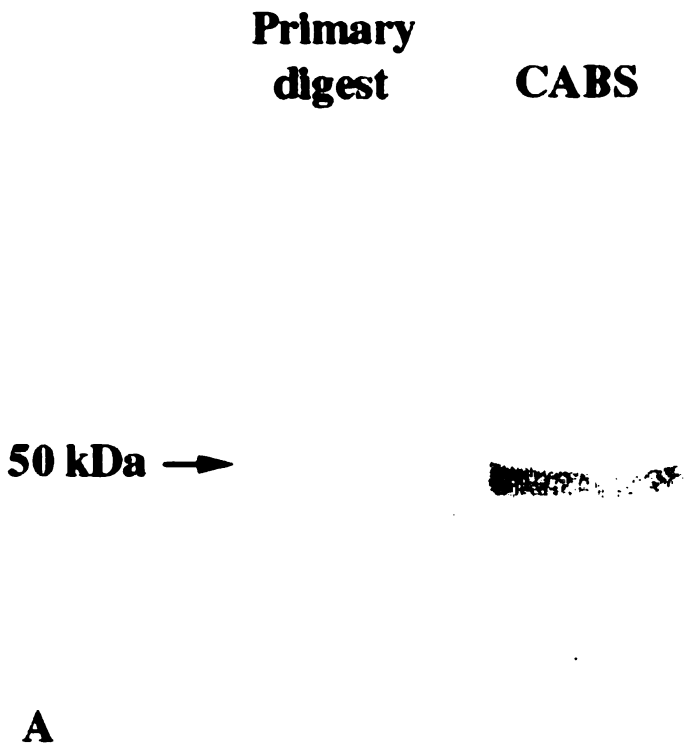



**Figure 16.**
**IHC analysis of CAB selected cells (20X) reveals intercellular upregulation of e-cadherin with increasing calcium concentration.**
(A) CAB cells selected and grown in KGM-2 + 0.05 mM calcium. (B) CAB cells selected and grown in KGM-2 + 0.05 mM calcium, and then changed to the same media with 0.15 mM calcium. (C) CAB cells selected and grown in KGM-2 + 0.05 mM calcium, and then changed to the same media with 1.0 mM calcium.




A

**Figure 17.**
**Western blot of CAB cell lysate selected in KGM-2 + 0.05 mM calcium with an anti-ameloblastin antibody.**
(A) Positive control and CAB cell lysate shows a positive reaction to ameloblastin with a 1:250 anti-ameloblastin peptide antibody. Secondary antibody was a 1:500 dilution of alkaline phosphatase labeled anti-rabbit antibody.
Positive control consisted of cells that were digested according to cell culture protocol, but never plated.

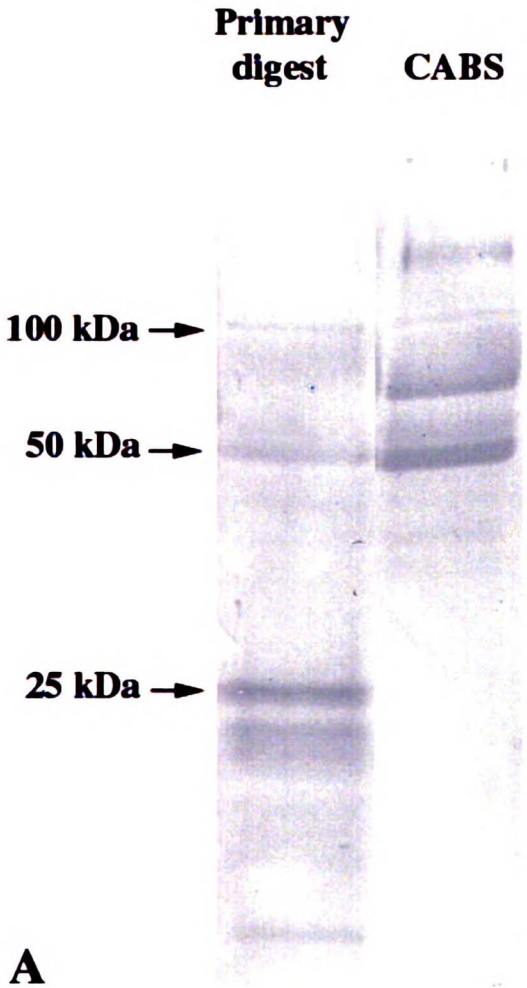



**Figure 18.**
**Western blot with an anti-amelogenin antibody of CAB cell lysate selected in KGM-2 + 0.05 mM calcium.**
(A) Positive control and CAB cell lysate shows a positive reaction to the higher molecular weight amelogenin aggregates. Primary antibody was a1:500 dilution of an anti-recombinant amelogenin antibody. Secondary antibody in both reactions was a 1:500 dilution of alkaline phosphatase labeled anti-rabbit antibody.
Positive control consisted of cells that were digested according to cell culture protocol, but never plated.

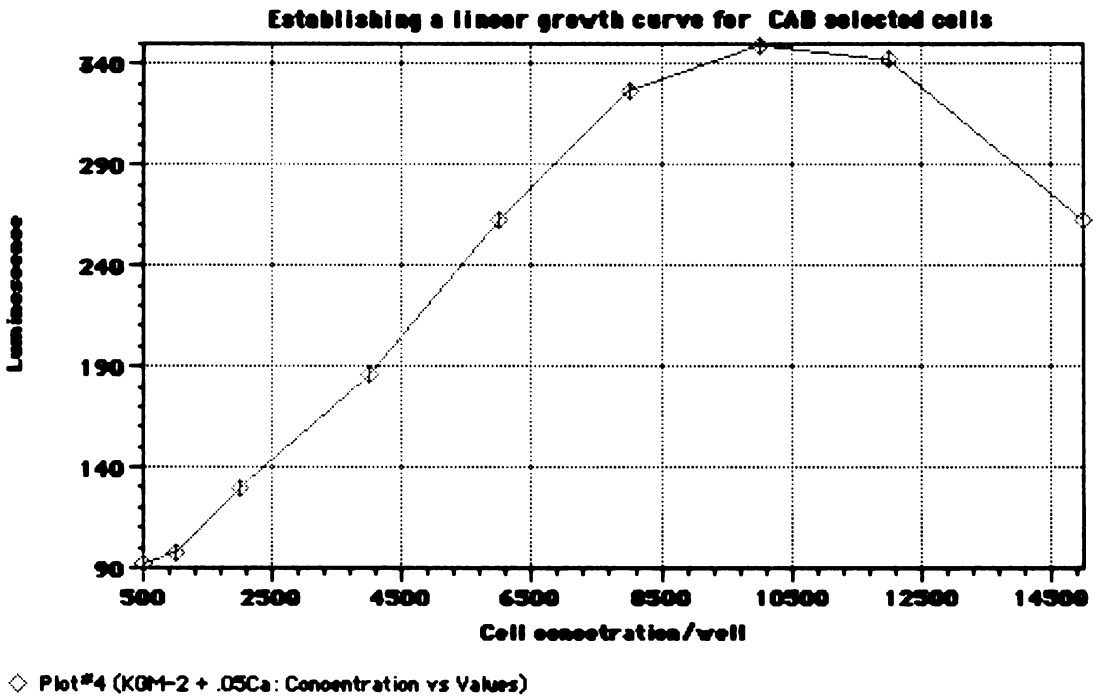



**Figure 19.**
**Proliferation of CAB selected cells in KGM-2 + 0.05 mM calcium.**
Cells plated at a concentration of 500-8000 cells/well showed linear growth within the parameters of this experiment.

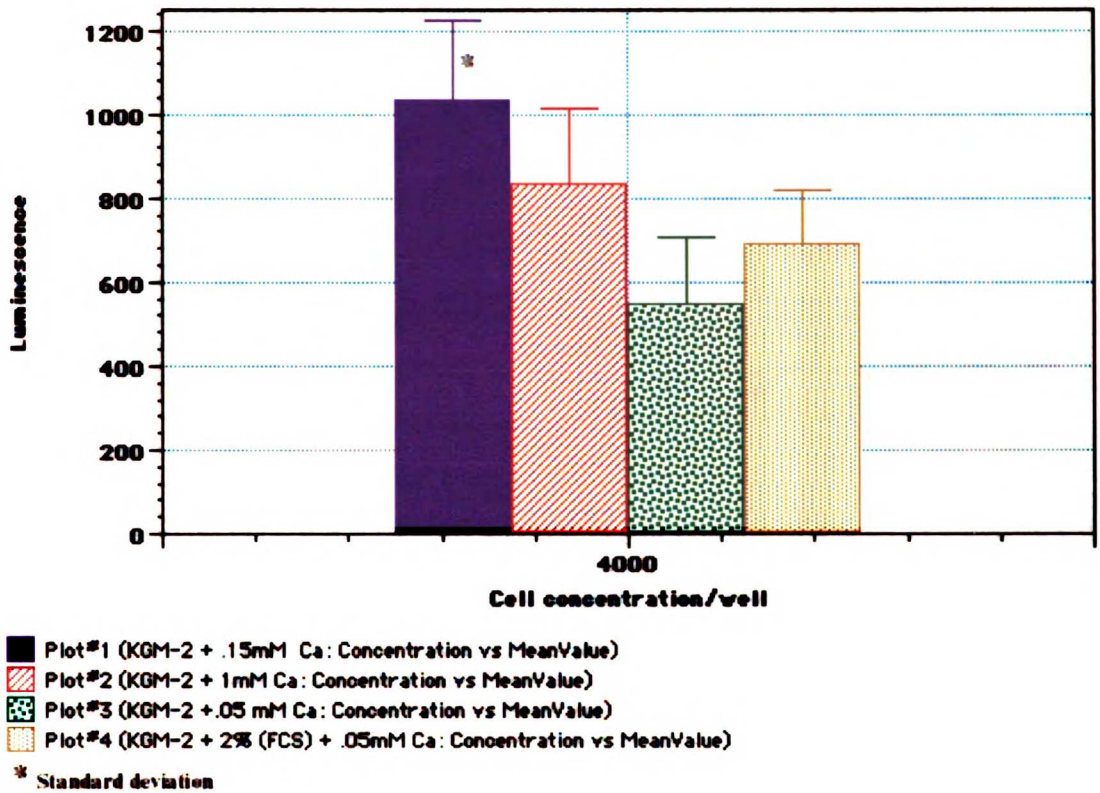



**Figure 20.**
**Proliferation differences of CAB selected cells in KGM-2 + 0.05 mM calcium with the addition of increased calcium or serum.**
Cells plated at a concentration of 4000 cells/well showed that calcium and serum effect the proliferation of CAB cells.

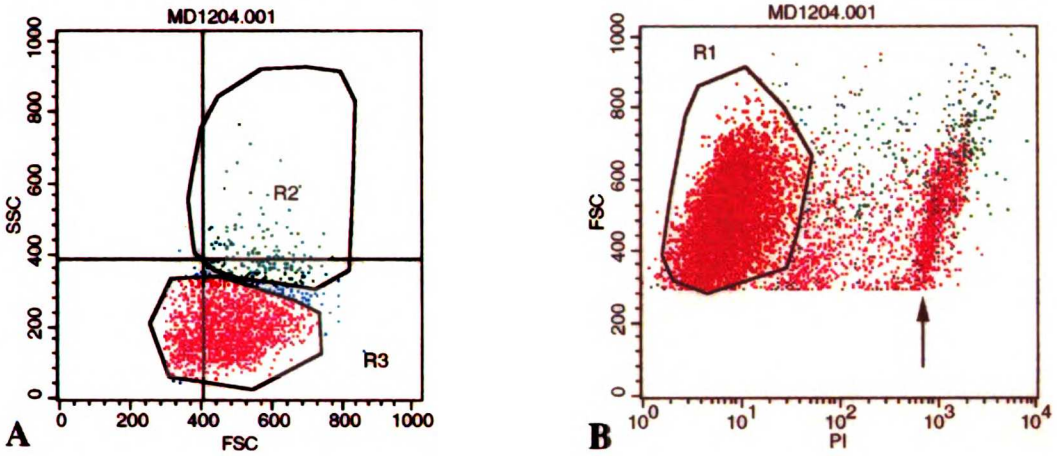



**Figure 21.**
**FACS analysis of a mixed a human enamel organ culture of mixed cell population grown in KGM-2 + 10% FCS + 0.05 mM calcium.**
(A) FACS analysis reveals only one cell population based on forward (FSC) and side scatter (SSC), although two phenotypic distinct cell populations exist (CABS and SPABS) in cell culture. B) Cells were labeled with propidium iodide prior to FACS showing the dead cells (arrow).

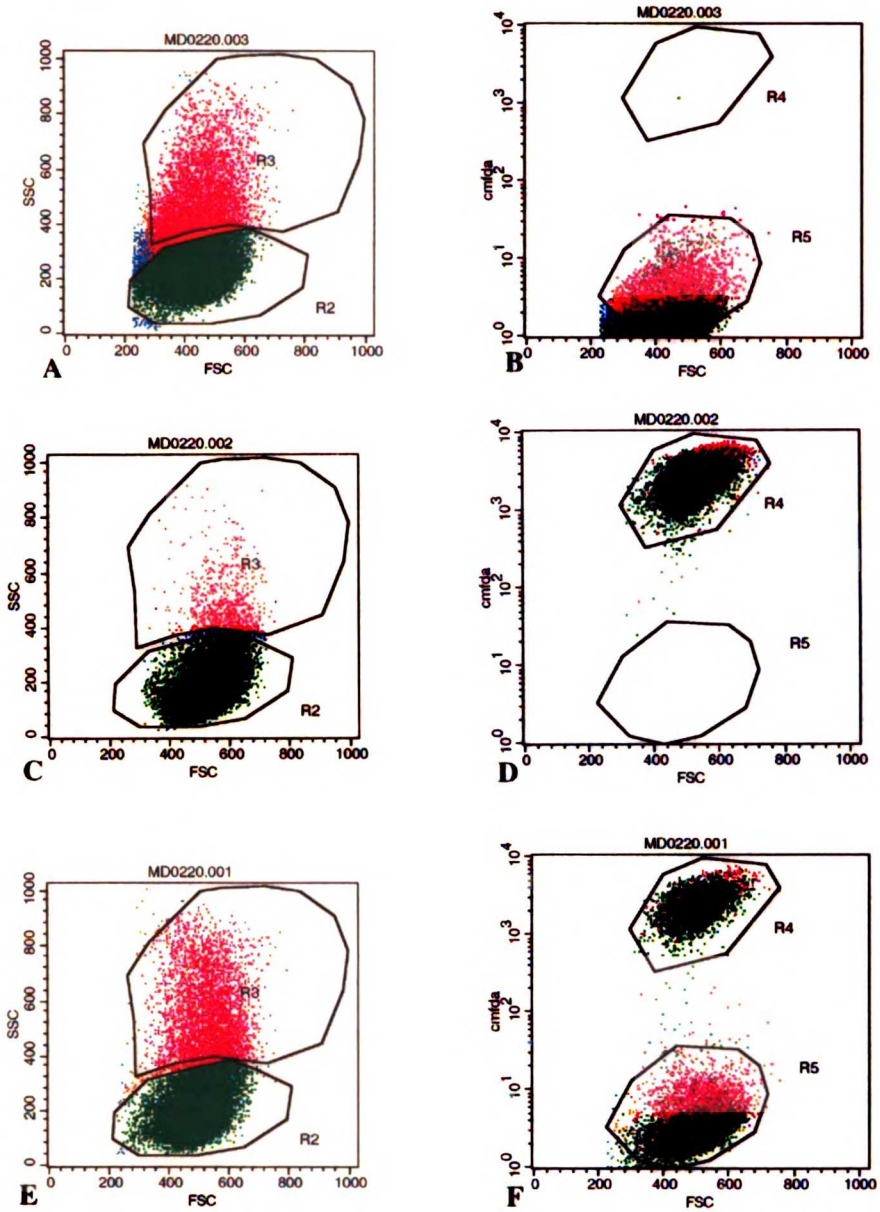



**Figure 22.**

**FACS analysis of two different enamel organ derived cell types (CABS and SPABS) alone, and mixed using a fluorescent label on the CAB cells.**

(A) The SPAB cell population reveals a similar pattern to the mixed cell population shown in figure 21, as revealed by FSC and SSC. (B) No fluorescence (CMFDA) is noted in the SPAB cells. (C) The CAB cell population reveals a similar pattern to the SPAB cells shown in A by FSC and SSC. (D) The CAB cells were labeled with a fluorescent cell tracker label and all show fluorescence (R4). (E) Labeled CABs from C were mixed with the unlabeled SPABS from A, and show a similar FSC and SSC pattern to the mixed cell population from figure 21. (F) Image reveals the ability to distinguish the labeled CABS (R4) from the unlabeled SPABS (R5).

This experiment confirms the difficulty of using FACS to separate the mixed cell population of CABS and SPABS without first using a cell specific label.

## Chapter 6. Primary culture and characterization of "ameloblast like" spindle shaped cells from human tooth germ.

### Introduction

The field of tooth development is moving forward to utilize advances in tissue engineering to reconstruct tooth formation in animal models. Two primary tissues, epithelium and mesenchyme, interact in an orchestrated event to form teeth. The mesenchymal component consists mostly of connective tissue which gives rise to pulp tissue. Pulp includes stem cells which differentiate into odontoblasts that give rise to dentin [1], a partly mineralized, partly organic tissue comprising the root and inner crown [2]. The epithelial component of teeth consists of an inner and outer dental epithelium that differentiates into cells such as stellate reticulum, stratum intermedium, and ameloblasts, which form the outside crown of the tooth. This outside crown forms enamel, a highly mineralized (96% by weight) tissue [2]. Recent advances in this area have shown the development of not only dentin, but enamel matrix in the shape of a tooth from an *in vivo* animal model [3, 4]. These studies have shown that when the cells of a developing tooth are separated by enzymatic digestion and then recombined in a matrix, the cells reorganize and form a tooth-like structure. Further advances in this area will require characterization of the individual cells that from this developing tooth.

The work I completed in chapter 5 describes my isolation and characterization of the cobble-stoned shaped epithelial cells (CABS) from the human enamel organ. In this study I also identified another ameloblast-like cell with a spindle shape (SPABS). The SPABS had phenotypic characteristics that more closely resembled fibroblasts, yet Kukita and co-

workers revealed these same shaped cells derived from rat incisors stained positive for amelogenin by immunohistochemistry [5]. The purpose of the studies described in this chapter is to further isolate and define the SPAB cells, and characterize these cells to determine if they did produce tooth specific mRNA and proteins.

## Materials and Methods

*Selection of primary SPAB enamel organ derived cells:*

Tooth germs from human fetal abortus specimens were isolated, washed in PBS, and digested with collagenase/dispase in PBS (1mg/ml) for 1hour at 37°C. The developing germs were isolated from many specimens with the average age of the specimen at approximately 18-21 weeks. The tissue was then collected, centrifuged at 2500 rpm for 5 minutes and washed with PBS. Digestion was then done with STV (0.05% trypsin, 0.025% versene, in saline A), for 5 minutes at 37°C. The cells were then centrifuged and washed as above, and 5 $X10^4$ cells were plated on a 100 mm Primaria (Becton Dickinson) tissue culture plate. Cells were cultured in KGM-2 + .05 mM $Ca^{2+}$ media for two weeks, and then changed to KGM-2 + 2% FCS + .05 mM $Ca^{2+}$ for subsequent passages. This method selected for the SPAB cells.

*Clonal Dilution*

Human SPABS selected by the above method were grown to passage 4 and digested in STV (0.05% trypsin, 0.025% versene, in saline A), for 10 minutes at 37°C. KGM-2 +2% FCS + .05 mM $Ca^{2+}$ (0.5 mls) was then added to inhibit the trypsin. The cell suspension

was centrifuged for 5 minutes at 2000 RPM. The pellet was then washed in PBS and centrifuged again for 5 minutes at 2000 RPM. The cells were then reconstituted in 1 ml of KGM-2 +2% FCS + 0.05 mM $Ca^{2+}$ media and counted using a hemocytometer. The cells were diluted to a final suspension of 50 cells in 19.5 mls. Two hundred ul of this passage 4 SPAB cell dilution was added into each of 96 well plates with approximately 50 cells/96 well plate.

**Biochemical analysis**

*RT-PCR:*

Culture conditions were used to select for SPAB cells. These cells were washed with PBS twice, and 600µl cell lysis buffer was added to the plates. The cells were then scrapped with a cell scrapper into a 1.5 ml eppendorf tube and total RNA was isolated using a SNAP total RNA isolation kit (Invitrogen). The mRNA was reverse transcribed into cDNA which was then used in various PCR reactions to further characterize the genotype of the cell populations. PCR primers are described below in table 1.

| |
|---|
| AMB H 333 U 5' AGGCCAAGAGAACATGAAACT 3'<br>AMB H 887 L 5' CTGGAAACATGGCTCCATAG 3' |
| AMG 03 U 5' GGCTGCACCACCAAATCATCC 3'<br>AMG 04 L 5' CCCGCTTGGTCTTGTCTGTCG 3' |
| ENAM H 3189 U 5' GCT AGCATGACCCTTTCCTGAAG 3'<br>ENAM H 3710 L 5' GAATTCCTAGCCTGAAGTAGTAAGCA 3' |
| **Table 1. PCR primers used for genotyping SPAB cells**<br>Ameloblastin (AMB), amelogenin (AMG), enamelin (ENAM). |

Positive PCR products from the ameloblastin and amelogenin specific primer reactions were cloned into a vector using a TOPO cloning kit (Invitrogen, LaJolla, California), and a positive colony picked for growth and plasmid purification using a Wizard PLUS plasmid purification kit (Promega). The plasmid was then sent for sequence analysis (Elim Biopharamceuticals, Hayward, California). This experimental approach was described in more detail in chapter 2.

*IHC:*

Collagen coated 8 chamber slides were prepared by pre-incubating the slides with 10ug/ml of collagen type 1 (Vitrogen 50, Cohesion, Palto Alto, CA, USA) at 37$^0$C for 1-3 hour before. The excess collagen was then removed and the dishes allowed to dry. SPAB cells were grown for 1-5 days on the chambered slides and then fixed in 1% paraformaldehyde + 0.1% NP-40. The fixed cells were washed with PBS three times and incubated with primary antibody to recombinant human amelogenin for 1 hour at room temperature. The cells were again washed with PBS three times and incubated with fluorescent labeled secondary antibody for 30 minutes at room temperature. After three more washes the cells were imaged with fluorescent light microscopy.

*Western blot:*

SPAB cells grown on 100 mm tissue culture dishes were digested with STV, centrifuged, and washed with PBS. The cells were then resuspended in SDS loading buffer and boiled for 10 minutes. The cell lysate was centrifuged and the supernatant loaded on a 12% polyacrylamide gel for SDS-PAGE. The separated proteins were stained with coumassie for analysis or transferred to a nitrocellulose membrane for western blotting. For transfer

a 0.75mm SDS polyacrylamide gel was transferred to a nitrocellulose membrane for 1 hour 15 minutes at room temperature. The membrane was then stained with Ponceau S to evaluate transfer efficiency and blocked overnight in 2.5% nonfat dried milk and 0.1% tween in TBS. The membrane was then washed 3 times in TBS for 5-10 minutes and incubated with primary antibody (anti-bovine peptide ameloblastin or anti-human recombinant amelogenin) at room temperature on a rocker for 2 hours. The membrane was then washed three times for 5 minutes each in TBS and incubated with secondary antibody labeled with alkaline phosphatase for 2 hours at room temperature. The membrane was again washed in TBS and stained with Sigma Fast BCIP/NBT (Sigma, St. Loius, MO, USA).

*Proliferation assay:*

The cells were digested in STV for 5-10 minutes at 37$^0$C, centrifuged at 2000 RPM for 5 minutes, washed with PBS, and spun again for 5 minutes. The cells were then reconstituted in KGM-2 media with 0.05mM $Ca^{2+}$ and counted using a hemocytometer. Before plating the cells the 96 well plates were first prepared by adding 50 ul/well of type 1 collagen (10ug/ml) for 1 hour at 37$^0$C. The cells were then plated in a black 96 well plate (Greiner, USA), at 4000 or 6000 cells/well and incubated in a humidified atmosphere at 37$^0$C for 24hours. A cell proliferation ELISA, BrdU (chemiluminescence) kit (Roche, USA) was used to examine the proliferation rate of the cells under different experimental media conditions. Briefly, after plating the cells (24hrs), different experimental media was added to the wells (KGM-2 + 2% FCS + 0.05mM $Ca^{2+}$, KGM-2 + 0.05mM $Ca^{2+}$, KGM-2 + 0.15mM $Ca^{2}$, or KGM-2 + 1.0 mM $Ca^{2+}$) at a concentration of

100 ul/well. The cells were further cultured in experimental media for 24 hours. The cells were then labeled with 10 ul/well BrdU added into the experimental media (10uM final concentration BrdU), and incubated at 37$^{0}$C for 2 hours. The labeling medium was removed by dumping and tapping the 96 well plate. The cells were then fixed and denatured by addition of 200 ul/well FixDenat solution (Roche, USA), and the cells incubated for 30 min. at RT. The solution was dumped and the bottom of the plate tapped, and anti-BrdU-POD working solution was added to the wells (100 ul/well) and the cells were incubated for 90 minutes at RT. The working solution was dumped and the cells were washed three times with 200ul/well washing solution. Substrate solution 100ul/well was then added to cells, the plate shaken on a rocking platform for 3 minutes and read on a fluorometer (Spectramax, Geminixs Molecular Devices corporation, California, USA) at emission wavelength 425. This experiment was repeated in triplicate with 12 wells/ experimental condition. An unpaired t test with Welch correction was used for statistical analysis.

**Results**

Cells grown in KGM-2 media with a low calcium concentration initially select for CAB cells (Chapter 5), however when the same media was changed to contain 2% serum the spindle shaped cells quickly overtook the epithelial cell population, especially during passage where the spindle shaped cells adhere more readily than the epithelial cells. These cells could be maintained at confluence, and were able to be passed into new dishes for subsequent analysis. These SPAB cells also grew in increasing density with

the increase of calcium as described in chapter 5, however the focus of this chapter is on the characterization of the cells in a 2% FCS, low calcium media.

To determine the characteristics of the spindle shaped cell population, a cell line was established from a single cell through clonal dilution. Four wells out of the 96 well plate established by clonal dilution grew from a single cell. One of these clonally diluted cell lines was further expanded for analysis. These cells have maintained ameloblast like characteristics and are referred to as spindle-shaped "ameloblast like" cells (SPABS).

*RT-PCR:*

PCR performed on cDNA that had been reverse transcribed from isolated cellular RNA in SPABS revealed positive tooth specific gene PCR products. Three primer pairs each specific to a separate gene sequence of ameloblastin, amelogenin, and enamelin were used to analyze primary SPAB cells. The ameloblastin specific PCR primers generated a positive PCR product of 554 base pairs in SPABS as well as from cDNA using the human tooth germ library controls. The amelogenin specific PCR primers generated a positive PCR product of 383 base pairs using cDNA from SPAB cell types, as well as the human tooth developmental library control. This same type of analysis was done using an enamelin specific PCR primer pair with a positive base pair product of 521, again using SPABS and the human tooth developmental library cDNA control. (Figure 1). Both the ameloblastin and amelogenin PCR products were sequenced to confirm the correct sequence. The enamelin specific primer product was not confirmed through sequence analysis.

*IHC:*

SPAB cells stained positive for amelogenin. The staining pattern of amelogenin in the SPAB cells was cytoplasmic. Control cells incubated with IgG isolated from pre-immune serum was negative (figure 2).

*Western:*

Western blot analysis revealed a positive staining pattern for two tooth specific proteins (ameloblastin and amelogenin) in the SPAB cells. The positive control, which included cells from digested tissue, (according to the cell culture protocol) showed similar positive staining. In SPAB cells the anti-ameloblastin antibody used in Western staining revealed three distinct bands at 50, 52 and 62 kDa. The positive control revealed a similar staining pattern, however sometimes lower bands at 48 and 46 kDa were also seen (figure 3). In the positive control the anti-amelogenin antibody revealed the main lower molecular amelogenin band at 25 kDa along with the higher molecular weight aggregates, however only higher molecular weight bands of amelogenin were seen in the SPAB cells (figure 4).

*Cell proliferation:*

Proliferation assays were first completed using multiple cell concentrations to determine a linear growth curve for the SPAB cells with and without serum (figure 5). These assays showed that a cell concentration of 4000 and 6000 cells/well worked well within the parameters of these experiments. The SPAB cells exhibited increased proliferated in

KGM-2 +(2%S) +.0.05 mM calcium compared to the same media without serum. These cells proliferated at a rate almost twice that of the same cells in serum free media (figure 6). The increased rate of proliferation in the serum containing media was statistically significant at both cell concentrations with a $P<0.0001$.

**Discussion**

Previous cell cultures derived from tooth germs have mentioned the formation of spindle like cells, fibroblast like cells, or mesenchymal cells [6-8]. Most of these analyses have regarded these cells as some type of contamination to the epithelial ameloblasts. One study, however, showed that the spindle shaped cells (in serum free media) were positive for amelogenin staining as detected by immunohistochemistry, while the cells (named as CAB in this dissertation) were not [5]. In my study I was able to selectively grow the SPAB cells to characterize these cells for expression of ameloblast specific mRNA and protein.

I used a clonal dilution strategy to successfully expand SPAB cells from single cells plated into a 96-well tissue culture plate. These cells showed positive expression of amelogenin, enamelin and ameloblastin by RT-PCR analysis. Further analysis by Western blot showed that amelogenin and ameloblastin protein were translated. The staining pattern observed by Western blots was similar to that found in CABS (Chapter 5). The initial characterization of these cells has been achieved, however future work is

necessary including the use of pre-immune controls, and the addition of peptide or protein to the Western blot reactions to confirm the specificity of the bands.

Early work on these enamel proteins has designated them as ameloblast specific [2]. Recently, "enamel" proteins have in fact been found in other areas of the teeth. Ameloblastin has been isolated to pre-odontoblasts, and cementoblasts [9, 10], while amelogenin has been implicated in root development and bone formation [11] (personal communication, Dr. Jim Wright). There is much more to these "enamel" proteins than can be explain by what is found currently in the scientific literature. Therefore, further studies to specifically localize the SPAB cells in the developing tooth will be needed to confirm that these cells are derived specifically from the enamel organ.

I used immunohistochemistry to localize amelogenin to the cytoplasm of these SPAB cells. This analysis coincides more closely to what one might expect from enamel proteins that have signal peptides designating them for secretion [2]. This result is in contrast to that found in the tooth germ cobble stoned ameloblast-like cells (CABS) where amelogenin staining was localized to the nucleus (Chapter 5). Further studies are necessary to confirm these results and to determine how amelogenin protein functions in these two cell types.

I examined the proliferation of the SPAB cells in relation to serum and serum free media at a low 0.05 mM calcium concentration. The serum containing media had a definite proliferation advantage over serum free media. Serum containing media was also shown

to increase proliferation in CAB cells (Chapter 5). However, the greatest proliferation rate seen in CAB cells was due to 0.15 mM calcium, and not 2% serum. Further work needs to be completed in this area to determine if calcium has similar effects on the proliferation of the SPAB cells.

There are many different cell types in the enamel organ including stellate reticulum, stratum intermedium, and ameloblasts. The ameloblasts themselves undergo different stages of differentiation including pre-ameloblasts, secretory ameloblasts, transition stage ameloblasts and maturation stage ameloblasts (which include smooth and ruffle ended ameloblasts) [2, 12]. It is possible that the SPAB and CAB cells are from two different cell types from the enamel organ, or from different stages of ameloblasts. The cytoplasmic staining pattern of amelogenin protein found in SPAB cells would be more indicative of secretory ameloblasts, while the nuclear staining pattern identified in CAB cells could be more indicative of a pre-ameloblast. Kukita and coworkers hypothesized that the CAB cells differentiated into the SPAB cells after stimulation by calcium [5]. They based this hypothesis on the increased number of SPAB cells and decreased number of CAB cells in culture after the addition of calcium. This hypothesis, however, did not coincide with my own results. Rather I found that a higher calcium concentration in the media led to a selective advantage of the SPAB cells leading to an increased number of these cells, and led to a change of monolayer CAB cells to stratification of these cells in culture (Chapter 5). Although calcium may not cause CAB to SPAB cell differentiation as hypothesized by Kukita and coworkers, calcium may lead to further differentiation of each cell type individually or another signaling factor could be involved. Further studies

are necessary to determine what molecule(s) lead to the differentiation of these two cell types.

## Conclusion

I have defined a cell culture system to select for a spindle shaped cell population that seems to have ameloblast characteristics. To ensure that the cells were a pure population I isolated the SPAB cells from subpopulations derived from a single cell. This subpopulation produced enamel proteins, which were detected by RT-PCR, immunohistochemistry, and Western blot analysis. These cells also showed a cytoplasmic staining pattern for amelogenin, typical of a secretory ameloblast like cell. This analysis also solves the disparity in the literature regarding two different cell types that produce enamel proteins (SPABS, and CABS). Further studies are necessary to confirm the cells characteristics and to determine the *in vivo* phenotype of these two cell types to help determine what parent population of cells in tissue gives rise to each cell type.

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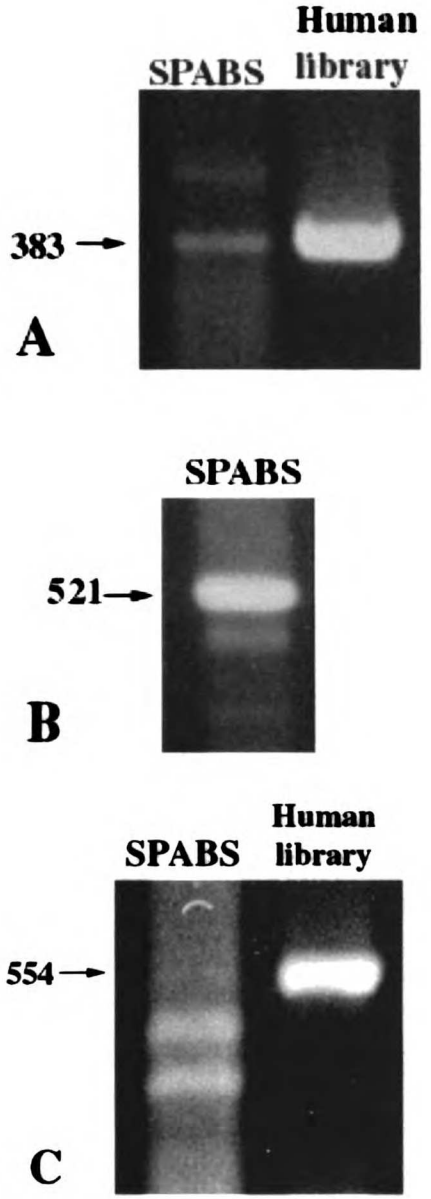


**Figure 1.**
**PCR of cDNA from SPAB cells.**
(A) Amelogenin specific primers were used to generate a positive PCR product of 383 base pairs in the cell cDNA and a human tooth developmental cDNA library control. (B) Enamelin specific primers generated the correct size of PCR product (551 base pairs) using SPAB cellular DNA. (C) Ameloblastin specific primers generated the correct size of PCR product (554 base pairs) using SPAB cellular cDNA and a human tooth development cDNA library.

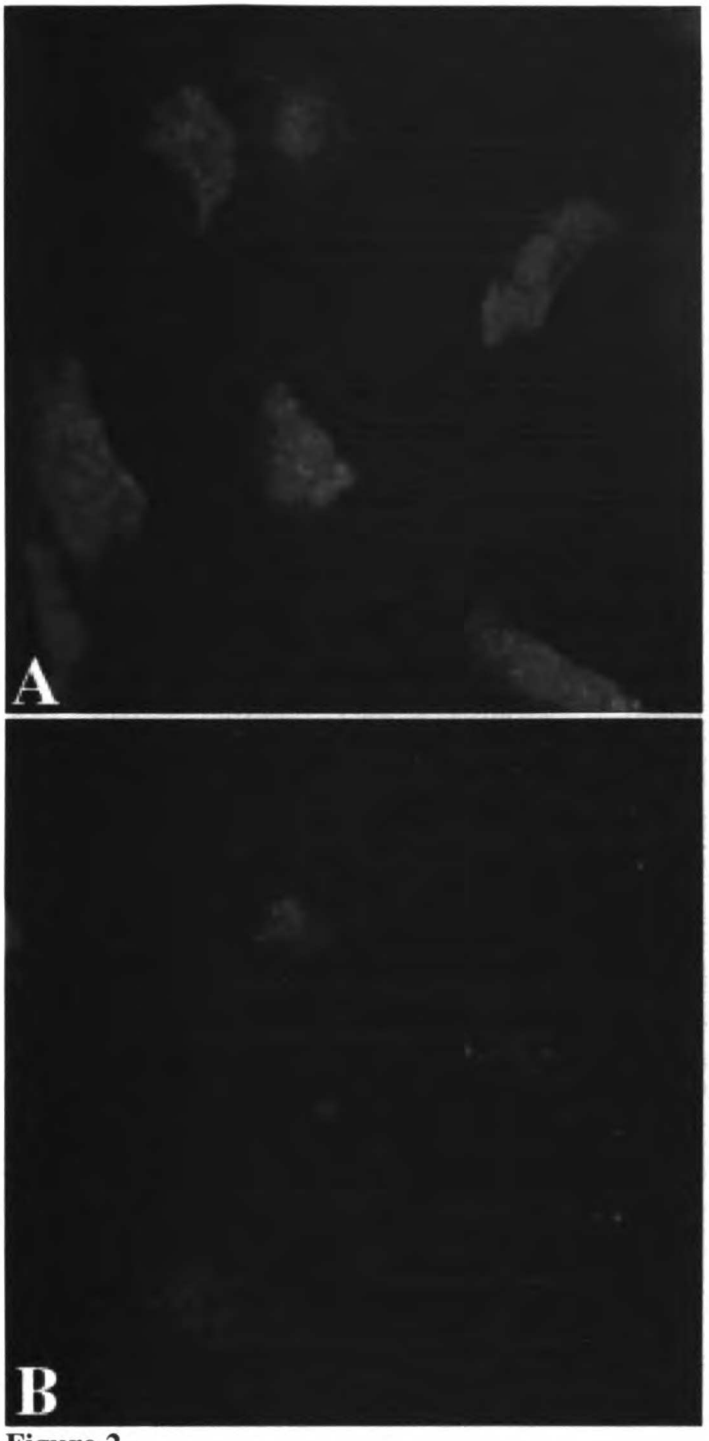


**Figure 2.**

**Fluorescent light microscopy image of SPAB cells selected in KGM-2 + 0.05 mM calcium and then grown in the same media with 2% serum.**

(A) Positive cytoplasmic staining pattern was observed in the SPAB cells with a 1:2000 anti-recombinant amelogenin antibody. (B) Negative staining was observed in the SPAB cells with a 1:2000 pre-immune serum from the same animal that the antibody was made. A 1:1000 dilution of fluorescent labeled secondary goat anti-rabbit antibody was used in A and B.

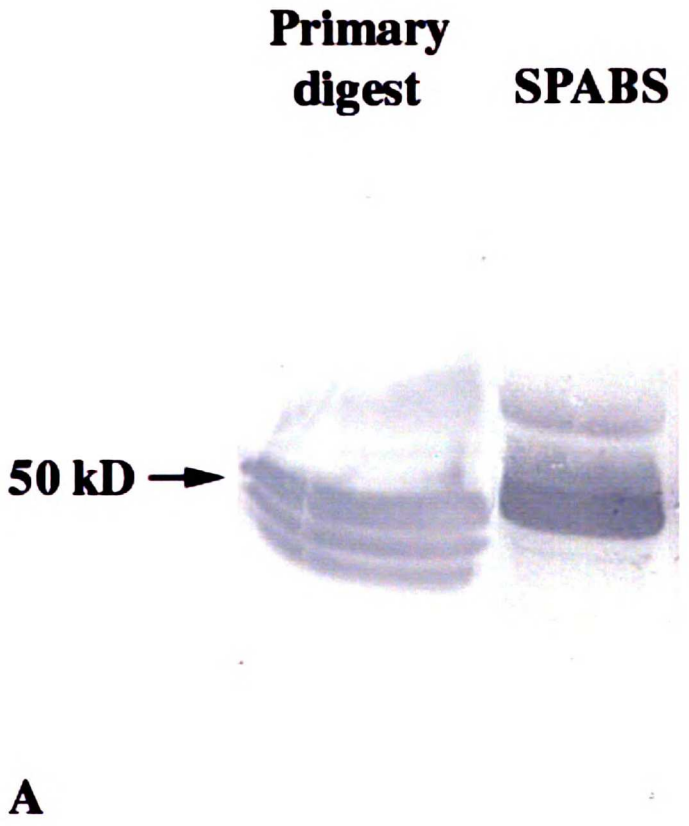


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**Figure 3.**
**Western blot with an anti-ameloblastin antibody of SPAB cell lysate.**
Cells were selected in KGM-2 + 0.05 mM calcium, then grown in KGM-2 + 2% serum + 0.05 mM calcium.
(A) Positive control and SPAB cell lysate shows a positive reaction to ameloblastin with a 1:250 dilution of anti-ameloblastin peptide antibody. Secondary antibody was a 1:500 dilution of alkaline phosphatase labeled anti-rabbit antibody.
Positive control consisted of cells that were digested according to cell culture protocol, but never plated.

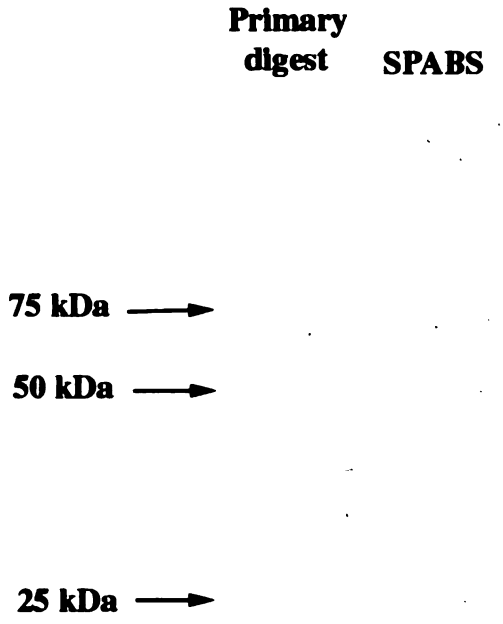


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**Figure 4.**
**Western blot with an anti-amelogenin antibody of SPAB cell lysate.**
Cells were selected in KGM-2 + 0.05 mM, then grown in KGM-2 + 2% FCS + 0.05 mM calcium.
(A) Positive control and CAB cell lysate shows a positive reaction to the higher molecular weight amelogenin aggregates. Primary antibody was a 1:500 dilution of an anti-recombinant amelogenin antibody. Secondary antibody was a 1:500 dilution of alkaline phosphatase labeled anti-rabbit antibody.
Positive control consisted of cells that were digested according to cell culture protocol, but never plated.

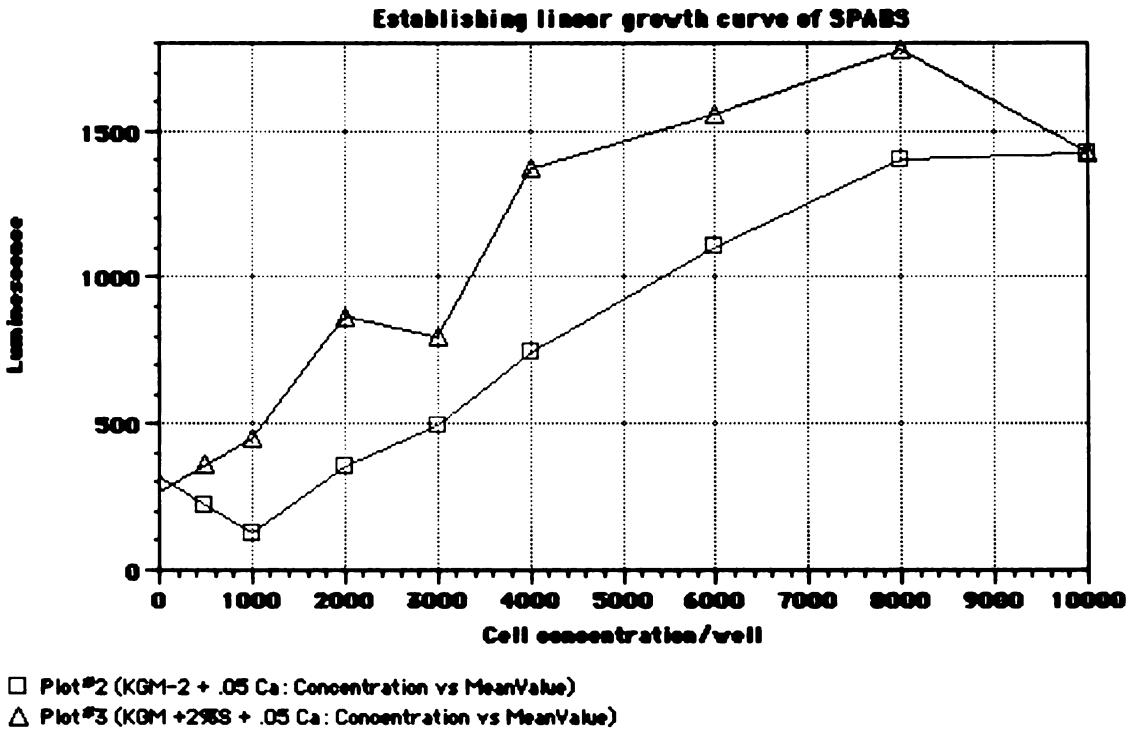


**Figure 5.**

**Proliferation of SPAB selected cells in KGM-2 + 0.05 mM calcium, and grown in KGM-2 + 2% (FCS) + 0.05 mM calcium.**

Cells plated at a concentration of 500-10000 cells/well. Linear growth was seen at a cell concentration of 1000-8000 cells/well within the parameters of this experiment.

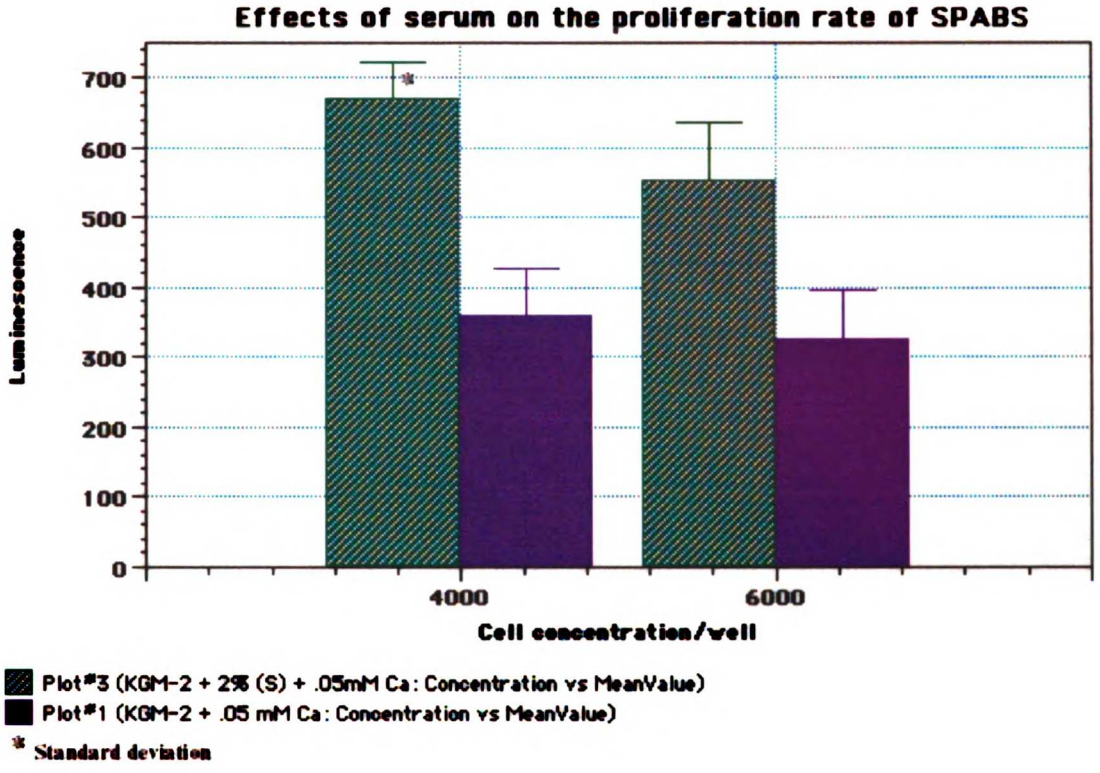


**Figure 6.**
**Proliferation differences of SPAB selected cells in KGM-2 + 0.05 mM calcium, and grown in KGM-2 + 2% FCS + 0.05 mM calcium.**
The addition of serum versus no serum was measured in relation to the proliferation effect on the SPAB cells.
Cells plated at a concentration of 4000 and 6000 cells/well showed that serum effects the proliferation of SPAB cells.

## Chapter 7. Conclusion and future studies

This dissertation contains a large body of work focused on the tooth specific protein ameloblastin. In summary, a new cDNA sequence was identified for this protein, a possible function has been determined regarding this protein, and although in these experiments a full-length nascent protein could not be purified from bovine enamel extracts, this protein has been identified in a newly established novel porcine and human tooth germ derived culture system.

Unlocking the bovine ameloblastin gene sequence

This dissertation is the first to identify the bovine ameloblastin cDNA and predicted amino acid sequence. These experiments led to the identification of a new previously unidentified splice variant for this tooth protein (Chapter 2), however further work is necessary to confirm the importance of this splice variant *in vivo*. The hypothesis was that the bovine ameloblastin gene sequence remained highly conserved between species. The specific aim to screen a bovine cDNA library for the ameloblastin sequence was used to confirm this hypothesis. The rationale was that by identifying a new ameloblastin cDNA sequence one could support stronger bioinformatics by looking at conserved motifs throughout the protein sequence compared to the two other known species (porcine and rat). This protein sequence would also be very useful in identification and purification of native ameloblastin protein from bovine enamel extracts that were available for analysis. This sequence information was important for the identification of predicted phosphorylation motifs that remain conserved between all identified species.

Another interesting finding was the highly conserved nature of the ameloblastin splice variants and the relation of the two different phosphorylation protein motifs predicted to be contained within each 15 amino acid deletion of the splice variants. It is well established that cellular signal processing depends on specific protein/protein interactions, and that protein phosphorylation plays a central role in cell communication by altering these protein interactions. Phosphorylation of a protein can have many different consequences for the protein. When –H is replaced with $-PO_3$ there are obvious size, charge and hydrogen bonding pattern alterations; all of these effects can and sometimes do lead to conformational changes in the protein itself. For example, calmodulin has been shown to have an electrophoretic mobility shift in the presence of $Ca^{2+}$, which is lost upon phosphorylation [1]. Likewise, myelin basic protein shows a higher ordered structure upon phosphorylation by circular dichroism. The functional significance of ameloblastin phosphorylation may take on different roles such as cell signaling, matrix structure, or mineral induction. For example, collagen mineralization is initiated by phosphoproteins [2]. By understanding the phosphorylation pattern of ameloblastin, we can then determine how phosphorylation is controlled through kinase activity and the role it plays in tooth development.

Defining possible functions for the ameloblastin protein

Although these experiments led to the identification and bioinformatic analysis of the bovine ameloblastin sequence, leading to plausible roles of phosphorylation, no functional work regarding this protein had been completed using a tooth development model. A hypothesis was developed that the highly conserved ameloblastin protein has a

role in tooth development. The specific aim to gain increased understanding of ameloblastin function during tooth development using a tooth organ culture system and a function-perturbing anti-ameloblastin antibody was used to test this hypothesis. The rationale was that by binding this protein to an antibody, the function would be diminished, and a phenotype could help establish the significance of this protein *in vivo*. The binding of the ameloblastin protein by the antibody led to an accelerated tooth development as determined by histological analysis through different tooth developmental stages. This analysis was the first to show a possible function of the ameloblastin protein, however, future studies with important controls are necessary both to confirm these results and further decipher the function of this protein.

One possible mechanism for the acceleration of tooth development by binding the ameloblastin protein includes ameloblastin's ability to bind calcium [3, 4]. Calcium has been shown to be a key regulator in the differentiation of keratinocytes [5] through an external calcium sensing receptor also found in the developing tooth ameloblasts [6, 7]. Ameloblastin may control calcium concentration at the site of ameloblast differentiation. In support of this hypothesis, it was shown that calcium can alter ameloblast structure (formation of intercellular bridges) and function (altered cell selection and proliferation (Chapter 6)). Markers specific to stages of ameloblast development, are needed to further define the role of calcium and ameloblastin in ameloblast differentiation.

To further test the hypothesis that the highly conserved ameloblastin protein has a role in tooth development a specific aim to purify the native ameloblastin protein from bovine

enamel extracts was used. The rationale was that in order to study the effects of tooth native proteases, and post-translational modification on the ameloblastin protein, it was necessary to purify the nascent ameloblastin protein. This purified protein was then to be utilized in the tooth germ culture experiments both as a control with the anti-ameloblastin antibody experiments (chapter 3), and in the media alone with the tooth germ culture experiments. A substantial effort was expended to purify the native ameloblastin protein from bovine enamel extracts (not reported in this thesis). This effort was not successful in that a major bovine serum albumin contaminant remained in the purified products thought to contain ameloblastin. However, these efforts in this project were helpful in characterizing MMP-20, leading to a co-authorship on a publication, which identified the functional domain of this enzyme [8].

Culture and characterization of tooth germ derived cells for analysis of ameloblastin

The interest in establishing a system that would allow the analysis of ameloblastin from a near native environment remained an important scientific question leading to the task of developing an "ameloblast-like" cell culture system. It was hypothesized that the cell culture system could be used to harvest ameloblastin from cells *in vitro*. In addition to establishing cells that would produce enamel proteins with post-translational modifications, this cell culture system was established with the aim of studying the function of the developing tooth in an *in vitro* (cell culture) and *in vivo* (cell transplantation) environment.

The subhypothesis was that by utilizing tooth germs for culture, a primary cell culture system could be established that would maintain some of the characteristics of ameloblasts. This hypothesis was tested by the specific aim of culturing and analyzing cells derived from porcine enamel organ tooth germs. The rationale was that by establishing a cell culture system, major advances would be made in this field, and further by establishing this culture in a serum free environment, serum would not remain a potential contaminant during purification of the ameloblastin protein. This technique was successful, though difficulties in passing and growing the cells for further analysis did exist (Chapter 4).

While culturing the enamel organ epithelial cells, another subhypothesis was developed that calcium controlled ameloblast differentiation. The development of the tooth through buccal infolding of keratinocytes [2], suggests that a mechanisms similar to those that control keratinocyte differentiation may also control ameloblast differentiation. The subhypothesis developed was that "ameloblast-like" cells could be established by controlling the amount of calcium in the media, similar to that found in keratinocyte cell cultures. This hypothesis was analyzed by using a keratinocyte growth media allowing one to easily alter the media concentration of growth factors (including serum), and calcium. The specific aims were to establish and characterize cobble stone shaped "ameloblast-like" cells, and to study the effects of calcium on the cobble stone shaped cells in culture. The rationale was that two laboratories had shown the cobble stone shaped cells to express positive signals to tooth specific proteins [9] [10]. Biochemical methods such as PCR, immunohistochemistry and Western blotting were used to confirm

the presence of tooth specific proteins in these cells, however as work on these cells continues further characterizations studies are necessary to confirm the "ameloblast like" characteristics. Studies of cellular response to different calcium concentrations showed altered proliferation and the upregulation of e-cadherin in response to calcium. This analysis of calcium effects on "ameloblast-like" cells, led to the development of methods to differentially culture primary cobble stone shaped "ameloblast-like" cells, and spindle shaped "ameloblast-like" cells. This analysis also led to a pure cell population from the primary culture of these cells contrary to earlier reports of mixed cell culture, again leading to major advances in this field [9, 11-13].

In conclusion, in this dissertation project, ameloblastin cDNA was analyzed to find a new splice variant for this gene, including bioinformatics data on plausible phosphorylation sites in this protein that could be controlled through splicing. A possible function for ameloblastin in controlling tooth development was elucidated, using tooth germ organ culture experiments. Two "ameloblast-like" cell types were separated with substantial characterization of these cell types. Clonal dilution was used to help confirm that each cell type is unique and produces tooth specific proteins, including ameloblastin, however, further characterization of these cells is necessary to truly identifiy these cells as "ameloblast like". It was shown that one of these cell types, the cobble stone shaped cells are regulated by calcium. These novel findings lay the groundwork for future studies that will include analyzing the role of ameloblastin in each cell type, as well as studies involving tooth development and tissue engineering with the individual cell populations.

**Future Studies:**

The tools and scientific evidence presented in this dissertation enable the continuation of ameloblastin research at a level unique to this laboratory. A human ameloblastin cDNA tooth developmental library has already been analyzed leading to further unique splice variants of this gene (data not reported). These splice variants will be used in transfection experiments to analyze the function of this gene on the "ameloblast-like" cells. Protein purification studies will also be developed to purify the ameloblastin protein from the "ameloblast-like" cells transfected with the ameloblastin cDNA. Further analysis of the post-translational modifications of ameloblastin and potential interactions ameloblastin has with both the mineralizing matrix, and other molecules, is important to understand the process by which teeth develop and mineralize.

Analysis of ameloblastin's post-translational modifications include its predicted phosphorylation. Further studies will include analysis of the role(s) of the splice variants of ameloblastin, specifically including the role these splice variants have in controlling sites of phosphorylation. These splice variants will also be studied using the tooth germ culture experiment for functional analysis, by using antibodies specific to these highly conserved 15 amino acid deletions reported in chapter 2.

Further studies are warranted to determine the differences between these "ameloblast-like" cells, including the parent cell population from where the cultured cells were derived. This analysis will include the transplantation of these cells into nude mice to examine the phenotype of these cells in an *in vivo* environment. These types of assays

will include not only the analysis of the epithelial phenotype from the "ameloblast-like" cells, but the addition of tooth germ mesenchymal cells to understand epithelial/mesenchymal interactions involved in tooth development.

In addition to looking for markers of differentiation, and markers specific to each "ameloblast-like" cell type these cells will be analyzed by examining the effects that various growth factors have on the regulation of these cells. This growth factor regulation could include what controls the expression of these enamel proteins such as ameloblastin. The culture of the ameloblast cells are a powerful tool that we have just begun to exploit in the pursuit of science, which will lead to further understanding of the developing tooth.

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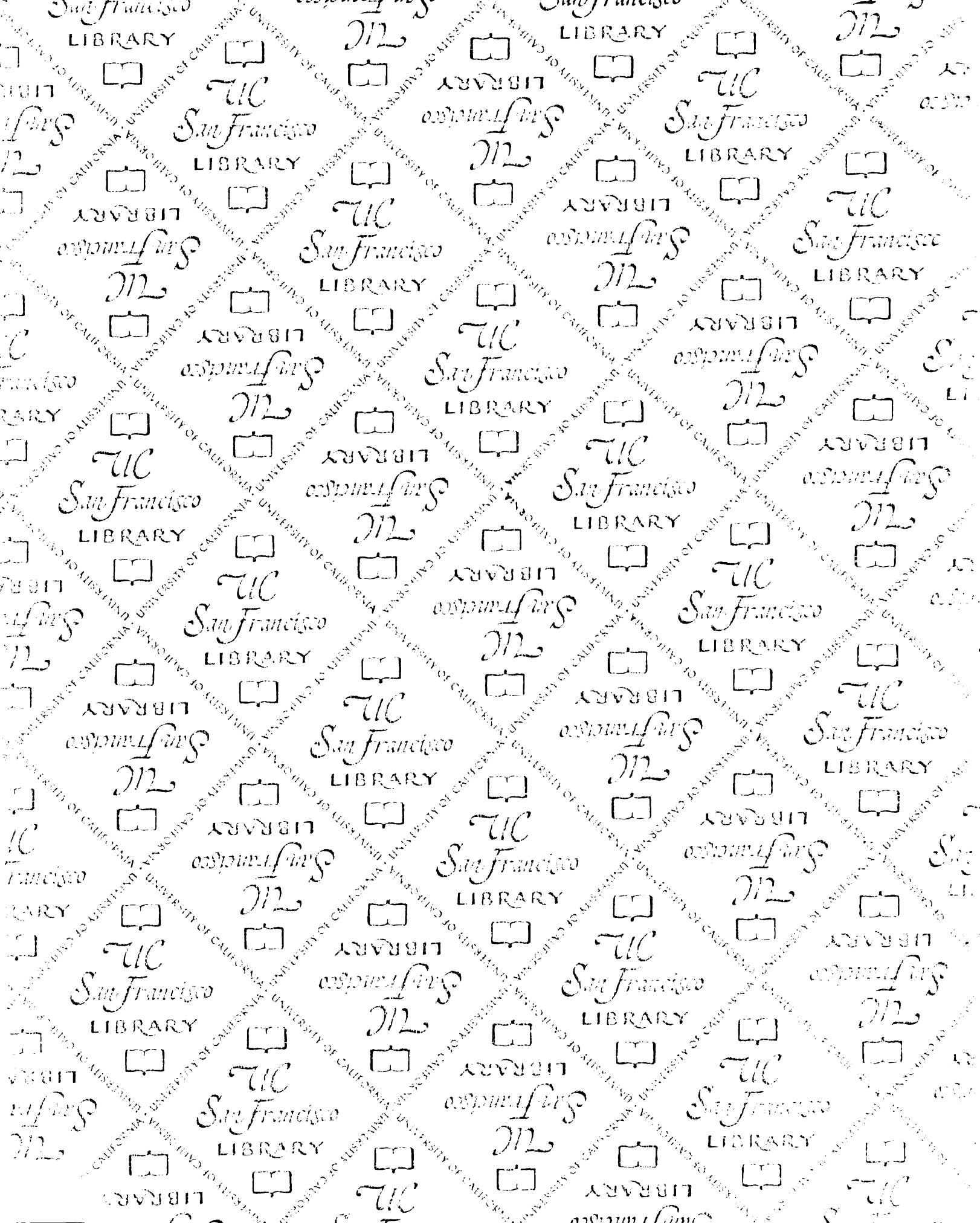
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