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Immunolocalization of gene products
responsible for Amelogenesis
Imperfecta and Dentinogenesis
Imperfecta in mice

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BOSTON UNIVERSITY

HENRY M. GOLDMAN SCHOOL OF DENTAL MEDICINE

THESIS

**IMMUNOLOCALIZATION OF GENE PRODUCTS RESPONSIBLE FOR
AMELOGENESIS IMPERFECTA AND DENTINOGENESIS IMPERFECTA
IN MICE**

by

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Dedication:

My thesis and every success that I have achieved is dedicated to the soul of my deceased father who have been in academia as a Lecturer in the electronic engineering department at King Abdulaziz University for over thirty years.

His support during his life, motivation, love and caring are what brought me to reach the individual I have become today. He is long lasting words are still the guiding lights to any path I may concur.

Also I would like to dedicate such an achievement to my loving mother for all the warm environment that I was raised up in and for her endearing care until this moment.

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ABSTRACT

Healthy tooth formation is crucially dependent on normal development of enamel and dentin. Any deviation from norm could lead to serious effects on the teeth function.

Amelogenesis Imperfecta (AI) and Dentinogenesis Imperfecta (DGI) are genetically inherited conditions that affect the teeth formation. Thus is imperative to investigate the genes and proteins that contribute to these conditions. Some of the known proteins that play a role in amelogenesis include AMELOGENIN (AMLEX), KALLIKREIN 4(KLK4), FAMILY WITH SEQUENCE SIMILARITY 83H (FAM83H), WD REPEAT-CONTAINING PROTEIN 72 (WDR72) and DENTIN SIALOPHOSPHOPROTEIN (DSPP).

The purpose of this research project was to investigate the expression/localization pattern of gene products which are known to be causative for Amelogenesis Imperfecta and Dentinogenesis Imperfecta. The study was carried out using mouse heads which were fixed, demineralized and paraffin-embedded. Samples were then sectioned and immunohistochemical analysis was performed with various enamel/dentin protein antibodies.

The data showed the following results:

KLK4 showed immunoreactivity mainly in ameloblasts and in the pulp, DSPP showed immunoreactivity in dentin, in the pulp and in the epithelial cells on one location as indicated by the arrow in figure 3 of the tooth cross section, FAM83H has a faint immunoreactivity identified in the ameloblasts, WDR72 showed weak immunoreactivity in the ameloblasts and AMELX showed immunoreactivity on the enamel and the ameloblasts.

In conclusion these findings were supported by previous studies and conveyed the validity of IHC experiments in locating these proteins in odontogenic tissues.

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Introduction:

Enamel and dentin are the major structural components of teeth. Their intact formation and development largely determine the proper function of a tooth and, subsequently, a healthy oral cavity. Tooth development is dependent on several stages of biochemical activities that are the result of the integration of various elements, for which each has a role in different stages of development. ¹

Ameloblasts and odontoblasts are important key players in the biochemical, physiologic formation and development of healthy enamel and dentin.

Amelogenesis:

Enamel is the hardest tissue of the human body, having the most inorganic composition. Enamel crystals measure 85% inorganic by volume². Additional research has found a percentage of less than 1% for organic material in fully mature acellular enamel. ³This unique feature of a high content of inorganic material allows the teeth to survive in a harsh

oral environment, withstanding different harmful conditions, including masticatory forces, chemical and other harmful stimuli, along tooth life.¹

Amelogenesis is defined as the biochemical, physiological process of enamel formation. It is divided into various stages that can be summarized as the pre-secretory stage, secretory stage, and maturation stage. Ameloblasts undergo different morphological changes to adapt to specific functional demands and requirements at each stage. After teeth erupt, the ameloblasts lose their function and no regeneration or even repair is possible after this point.

Briefly, during the initial pre-secretory stage, ameloblasts gain their phenotype and polarity in addition to assemble organelles that contribute to protein synthesis preparing for the secretory stage. Tooth formation starts by the crown shape appearing to be defined early in the bell stage of a tooth development. At this point the inner enamel epithelium cells and dental papilla are separated by a basal lamina. The cells of the inner enamel epithelium at this point are cuboidal in shape and have scattered organelles in the cell cytoplasm.¹ Those cells tend to get longer during differentiation and all organelles start to arrange, giving an indication of

functional changes as the nucleus shift proximally, the rough endoplasmic reticulum increases significantly, and the mitochondria tends to accumulate in the proximal region. In addition, the Tomes' processes form on ameloblasts distal extremities and these changes make the cell polarized and lose the ability for further division. ¹Later, at the secretory stage, ameloblasts secrete enamel proteins which play a role in forming the general outline of future enamel, allowing for its full thickness formation. In coordination with secretory stage activity, the ameloblasts have an extensive Golgi apparatus surrounded by rough endoplasmic reticulum, reflecting the process of enamel protein secretion. Secreted granules are emitted from the distal Tomes' processes against the mantle dentin to form rod-less enamel that is followed shortly afterward by mineralization. ¹at this time ameloblasts are kept in an organized alignment manner by junctional complexes, which have an important role in amelogenesis by keeping ameloblasts tightly held in place and also controlling the transport of substances. As the initial enamel layer of deposited ameloblast migrates, creating proximal and distal portions of Tomes' processes which looks, these geometric changes in the Tomes' processes explains the later rod/inter-rod pattern of the enamel crystals. The first proximal part of the ameloblasts near the junctional complexes

along with adjacent ameloblasts secretes the inter-rod enamel that is always deposited before the rod enamel, which is secreted from the face of the ameloblasts distal portion of Tomes' processes. This timing implies the role of the inter-rod outlining for deposition of the rod enamel. ¹Lastly, at the maturation stage ameloblasts organize the growth of inorganic mineral crystals and to follow a particular pattern. At the maturation stage enamel crystals grow in size, taking the space for enamel proteins and enamel water, although there is some secretion activity still in process. After that, ameloblasts undergo apoptosis which starts with removal of the organic materials and water, emptying a space for deposition of inorganic materials. This event in the ameloblasts cell life changes the ameloblasts into ruffled end shape cells. It has been found that this time for ameloblasts is responsible for substitution of the organic material with inorganic, once alteration in the permeability of the enamel organ is achieved. ¹

Enamel Matrix Proteins:

Enamel organic matrix is made up of a group of several important proteins. The most abundant protein in this group is amelogenin, which represents 90% of enamel protein. ¹The rest of the proteins in the enamel matrix other than amelogenin are such as enamelin and ameloblastin,

which are the most studied two proteins of this group. ¹Other proteins secreted by ameloblasts are proteinases, such as matrix metalloproteinase or enamelysin (MMP20), which process newly secreted matrix proteins, and kalikern 4 (KLK4), which act as proteinase for the enamel protein at the maturation stage. ¹The presence of some enamel proteins in small amounts does not reflect the magnitude of production for such proteins, rather than its short half-life allows for less accumulation over long periods.

Dentinogenesis:

Odontoblasts are the most important cell type in dentinogenesis. These cells differentiate from ecto-mesenchymal origins due to influence from the inner enamel epithelium.

Odontoblasts are differentiated from the dental papilla, which later form the dentin.¹The cells of the dental papilla which will change to be the odontoblasts are in contact with the acellular zone. This acellular zone maintains a space between the cells of the dental papilla and the cells of inner enamel epithelium. Nevertheless, changes in dental papilla cells begin by gaining their phenotype by enlarging and elongating happens soon after the cell of the inner enamel epithelium reverse in polarity. The

sequence of events mentioned above indicates that the cells changing events may be attributed to signaling between the cells of dental papilla and the inner enamel epithelium at this stage. ¹After that the acellular zone between the cells of the dental papilla and the inner enamel epithelium seems to vanish, allowing the odontoblasts to occupy its space and be directly in contact with the ameloblasts which defferntiated from inner enamel epithelium.

Deposition of dentin begins when the first collagen is deposited by the odontoblasts into the extracellular matrix of the dental papilla, which are known as Knopf's fibers. These fibers contain collagen type 3.¹ As the odontoblasts continue to grow in size they secrete collagen type 1 in a parallel to the area known as dentinoenamel junction, and this primary dentin formed is known as "mantle dentin".¹ At the same time as collagen is secreted into the matrix, the odontoblasts start growing stubby processes. After that odontoblastic processes grow to form the known Tomes' fibers, it allows the cells to migrate away from the ameloblasts and towards the future pulp, creating odontoblastic tubules behind them. Dentinal tubules run through dentin in canaliculi from the dentin-enamel junction to the pulp, forming a distributional network of nutrients.

This demonstrates the path taken by the odontoblast to the pulp, which also meets other dentinal tubules in an anastomosing fashion.¹

Dentin mineralization process and patterns:

The first theories about dentin mineralization hypothesized that matrix vesicles were believed to grow and rupture to distribute crystallites clusters that fuse with crystallites from neighboring vesicles to create a continuous layer of mineralized matrix. It also added that formation of an inorganic mineralized matrix is created in a delayed manner to the deposited organic matrix of pre-dentin that comes between the mineralized matrix and the odontoblasts. At this point in the process, secretion of non-collagen matrix protein controls the deposition of minerals.¹ Recently more investigation showed that dentin mineralization starts as the collagen fibrils are packed and formed covalent bonds to hold its structure. The arrangement of this collagen fibrils form the scaffold for the mineralization process. Soon after the first nucleation of spherical mineral is deposited in between the collagen fibrils. The pattern of mineral deposition change from spheres to plates like shaped minerals arranged over each other.^{4,5}

The pattern of mineralization depends crucially on the rate of dentin formation. The globular pattern created by deposition of crystals in several

desecrates matrix directions continues to form until it fuses to form one large calcified mass. Deposition continues at different rates where the largest globule reflects a faster rate of deposition. In contrast, slow deposition of dentin shows a pattern with a more uniform mineralization and is referred to as “linear calcification”. Dentin is believed to have the same content and form in the crown and root, except for some reported differences hypothetically caused by the blending of collagen fibers of cementum and dentin on the root dentin.¹ Also, phosphoprotein is thought to be different in root dentin as it forms at a slower rate and has a different degree of mineralization.

The former mentioned process describes the formation and the pattern of the primary dentin, which is the most abundant form and is also called circumpulpal dentin. Secondary dentin starts to be formed after completion of root formation. It is formed from the same odontoblasts, forming the primary dentin only at a slower pace and has been shown to have less organization of dentinal tubules, also stained differently and has subtle demarcations that can histologically distinguish it from primary dentin. Tertiary dentin develops in response to injury or harmful stimuli to odontoblasts. The rate of dentin formation in tertiary dentin is largely dependent on the level of intensity of the harmful stimuli. The proportional

response is directly related and can be explained thus: As higher the magnitude of the stimulus the faster the rate of tertiary dentin deposition. The rapid pattern of formation leads to trapped cells in the newly formed matrix and distortion of the tubular pattern. ¹

Dentin Extracellular Matrix Protein (DECM):

Odontoblasts secrete the two major types of organic components that create dentin extracellular matrix protein, which are collagen and non-collagenous proteins. Collagen mainly consists of type 1 (approximately 86%) and other types, such as type 3, type 5 and type 6 are also found. Non-collagenous proteins are divided into osteonectin and the family of small integrin-binding ligand N-linked glycoprotein (SIBLINGS). SIBLINGS are the major proteins in DECM and include osteopontin (OPN), matrix extracellular phospho- glycoprotein (MEPE), bone sialoprotein (BSP), dentin matrix protein (DMP1), and dentin sialophosphoprotein (DSPP). ⁶

Among all of the previously mentioned proteins, the most abundant of them are dentin sialoprotein (DSP) and dentin phosphoprotein (DPP), which has been claimed to be a key role player of the dentinogenesis process, both are derivatives from cleavage processes of protein DSPP. ⁶

It was thought that DSPP expression is only shown in differentiating

odontoblasts, but a recent study showed expression in other tissue as in ear and bone.⁶⁻⁸

Amelogenesis Imperfecta:

The term Amelogenesis Imperfecta (AI) refers to the clinical conditions that affect tooth enamel formation without any association with other extra-oral tissues signs or symptoms.¹¹ It affects the enamel of nearly all the teeth in the affected cases.¹¹ It is considered to be a developmental issue that shows hypoplastic formation of the enamel, or even hypomineralization. It has a genetic origin and it is inherited in different ways that could be autosomal dominant, autosomal recessive, sex-linked, or even sporadic inheritance.¹¹

The prevalence of AI varies according to population, with a high prevalence in Sweden at 1.4:1000.¹² And in a lower numbers in Israel at 1:8000.¹³ and is even less common in USA at 1:14000.¹⁴⁻¹⁶

Classification:

The multiplicity of AI makes it complicated to achieve one system that can help define all different subtypes making it hard to find one ideal classification system. Some researchers have suggested classifications that rely on phenotype and clinical features, such as Witkop's

classification.¹⁷ Some other classifications that are formed to depend solely on appearance are not consistent in all cases and can be problematic.³ Other classification systems rely on the genetic mode of inheritance, which is thought to be useful in some cases. Recently, practitioners found that combining the pronounced mode of inheritance and the phenotype to be a promising method.¹¹

According to the phenotype, researchers classify the condition into mainly three types.^{18 17} First, the hypoplastic type shows enamel in a higher radio-opacity than dentin and the defect occurs due to impairment in ameloblast function, resulting in a diminished thickness of enamel and cavities on the tooth caused by opposition defect. This could be generalized or even localized in some other cases. The second is the hypocalcified type which manifested as normal thickness of enamel but shows defective mineralization process of the prism and rod of enamel crystals, which leads to enamel that can easily chip off a tooth. This type is considered to be the most common type with a prevalence of 1:20000.¹⁵ It shows enamel is lower with radio-opacity than dentin. The third classified subtype according to phenotype is the hypomaturation type that has normal enamel thickness, but an immature nature that suggests uncompleted removal of proteins leading to affected hardness and

transparency. In this type enamel and dentin has a similar level with radioopacity.¹⁸

Amelogenesis Imperfecta from a genetic perspective:

Witkop's classification has proceeded with the inclusion of genetic inheritance with the clinical manifestation to have more generalized and easy way to classify the condition.¹⁹ Witkop has shown that enamel defects can be distinguished in a carrier female in certain examination settings. This conclusion could imply that AI may have an X-linked dominant mode of inheritance. In addition, Winter and Brook showed that the phenotype of the normal thickness soft enamel that can be penetrated by dental probe is a feature of X-linked recessive hypo-maturation Amelogenesis Imperfecta.^{20 11} Also Haug and Ferguson et al examined a relatives of X-linked recessive AI cases. The authors found that common features that suggests genetic attribution to the conditions, including vertical linear ridges between normal enamel that has voids and wrinkles in every crown, relatively soft, abraded, or discolored enamel, and also enamel that shows a resistance of the cervical part of posterior teeth than anterior ones.²¹ Furthermore snowcapped teeth or Amelogenesis Imperfecta hypomaturation type were thought to have genetic involvement and in that sense it was studied by Witkop but he found that there was no

clear mode of inheritance. Later on Escopare et al, on the other hand, suggested that the snow capped tooth condition is X-linked.²² Further research by Witkop also added to the probability of autosomal dominant inheritance.¹⁶ Carwford and Aldred et al showed in their study that this condition can be autosomal dominant by demonstrating male to male inheritance.²³ Relying on previously mentioned researches and recent researches data Amelogenesis Imperfecta it was shown that the condition has different modes of inheritance. It can be autosomal dominant or recessive and it can be X-linked recessive. These modes of genetic inheritance make it possible to subdivide the condition into the following:

X-chromosome Linked Type:

Initial analysis studies of families with X-chromosome linked AI showed that the mutation corresponded with the *AMELOGENIN* locus (*AMELX*). Nowadays, there are 15 known mutations in the gene responsible for coding of *AMELOGENIN*, that are thought to be responsible for the hypoplastic and hypomineralized AI phenotypes.¹⁸ The phenotype ranges in males from normal enamel to extremely thin, local hypoplasia with no prism structure, or even retaining the amelogenin proteins. On the other hand, heterozygous women show vertical hypoplastic bands separated by normal enamel lines.¹⁸

Total loss of the amelogenin protein secretion causes the formation of enamel to occur in only a thin layer. This finding is supported by the fact that absence of amelogenin is not compatible with the formation of normal enamel crystallite size and normal prismatic architecture. In addition, Gibson et al found that very thin enamel is detected in *amelogenin* knockout mice which prove the involvement of X-chromosome linked gene in the development of AI. ^{18,24-26}

Autosomal Dominant and Recessive Inherited Types:

In theory, several autosomal candidates that have potential for association with AI, including: *AMELOBLASTIN (AMBN)*, *ENAMELIN (ENAM)*, *MATRIX METALLOPROTEINASE 20 (MMP20)*, *KALLIKREIN 4 (KLK4)*, *AMELOTIN (AMTN)*, *DISTAL-LESS 3 (DLX 3)*, *FAMILY WITH SEQUENCE SIMILARITY 83H (FAM83H)*, and *WD REPEAT-CONTAINING PROTEIN 72 (WDR72)*. ¹⁸

The causative genes that were found to have mutation causing various phenotypes are *ENAM*, *MMP20*, *KLK4*, *DLX3*, *FAM83H*, and *WDR72*. ¹⁸

No mutations have been found in the other theoretically potential causing genes previously mentioned. Autosomal dominant AI in some populations

is considered to be the most frequent form of AI, according to epidemiological studies.^{18,27}

Autosomal dominant AI (ADAI) has a broad-spectrum in terms of a clinical display. It has been difficult to relate the mutation to a specific phenotype and even more to pathogenesis, which is not clearly defined yet. Nevertheless, mutations in *ENAM*, *DLX3* and *FAM83H* are thought to be associated with the ADAI hypocalcified type.^{3,18,28,29} On the other hand, mutations in enamel protease genes, such as *KLK4* and *MMP20*, have been shown to occur in an autosomal recessive manner and are associated with the hypomaturation type of AI.^{18,28} Mutations in such genes cause the loss of protein or the production of proteinases that are not performing a known role in the mineralization process. Also the proteinases may be present but lacking their catalytic activities therefore it will result in an enamel that is not fully mineralized. The enamel matrix in this case is immature, as most of the protein remains and will be harnessed for enamel crystals growth due to the lack of function of these proteinases.^{18,30,31}

Dentinogenesis Imperfecta (DGI):

Dentinogenesis Imperfecta is an inherited developmental condition that occurs in the absence of any systemic disorder. It is also known as

hereditary opalescent dentin. Similar conditions can happen in conjunction with hereditary systemic bone disease called Osteogenesis Imperfecta (OI).¹⁷ It is the most common hereditary condition affecting dentin formation and mineralization. It also involves primary and permanent teeth and It is inherited as an autosomal dominant trait.

The first recognition of the disease is still a debatable subject; it has been suggested that Barret was the first to recognize this defect in 1882. It was first described as an enamel defect in a published report by Talbot and quoted by Witkop. The first to use the term 'hereditary opalescent dentin' was by Skillen, Finn, and Hodges describing brown teeth with a lacking pulp chamber and opalescent thin enamel that may be found in some cases, in other teeth weaker that have little resistance to wear and breakage.³²

Classification:

As a multifactorial process with different phenotypes, classification of Dentinogenesis Imperfecta is diverse and according to that, more than one system has been suggested. The most well-known classifications used in Dentinogenesis Imperfecta are Shields' and Witkop's classification.¹⁷ In 1973 Shields et al suggested classification into 3

divisions. The first, Dentinogenesis Imperfecta type 1 includes a family that has the defective dentin condition with the Osteogenesis Imperfecta as an autosomal trait.^{17,33} This condition was attributed to two genes that contribute to collagen type 1 encoding *COL1A1* or *COL1A2*.³⁴ Dentinogenesis Imperfecta type 2 is the most common type and includes families with a separate dentin defect which is inherited in an autosomal dominant pattern. The defect shows involvement of all dentition, has opalescent teeth with blue to brown discoloration, and has distinctive translucency which shows thin enamel that can be chipped away easily, leading to rapid attrition of the dentin. Radiographically, the teeth are bulbous with a constricted cervical part, thin roots, and obliteration of the pulp canal. Type 2 condition has 100% penetrance and variable expressivity. In some cases, enamel dysplasia was manifested, but it is likely caused by secondary defects.¹⁷ The defect is found to be caused by DSPP gene mutations.^{34,35} Dentinogenesis Imperfecta type 3, also known as Brandywine type as described by Witkop, is the rarest of all kinds and appears to be racial segregated in Maryland, USA and has an autosomal dominant trait.^{33,34} Despite the large clinical similarity in DGI type 3 and DGI type 2, these types are widely different radiographically, in which DGI type 3 presents the so-called “shell teeth” that is caused by the

dentin hypotrophy. The condition shows teeth having enlarged pulp chamber, which is poorly mineralized and widened root canals and ⁹teeth in DGI type 3 show multiple pulpal exposures. The same mutation in DGI type 2 found to be responsible for DGI type 3.

Purpose:

The purpose of this research project was to investigate the expression/localization pattern of gene products which are known to be causative for Amelogenesis Imperfecta and Dentinogenesis Imperfecta.

Materials and Methods:

Reagents:

For tissue fixation, formaldehyde, 10% (v/v) Aqueous Solution, RICCA Chemical (500ml) purchased from Fisher Scientific was used. For demineralization, EDTA from Sigma Aldrich was used E-5134. For sample preparation, Ethanol Flammables was used from Pharmaco-AAPER. For paraffin embedding of samples, preparation xylenes (Histological Grade) from Fisher Scientific (X3p-1GAL) was used. For blocking, hydrogen peroxide from Acros Organics and methanol cert (ACS/HPLC 4L) from

Fisher Scientific (A4524) were used. For washing, phosphate buffered saline, (PBS Powder) 10x1 Liter from MP Biomedicals was used. For blocking, the avidin/biotin blocking kit (SP-2001) from Vector Labs was used. For immunohistochemistry, biotinylated goat anti-rabbit IgG (BA-1000) from Vector Labs was used. A vectastain ABC elite standard kit (PK-4000) was purchased from Vector Labs. For staining of the secondary antibody, Peroxidase Substrate Kit DAB Vector Labs (SK-4100) was used. For Hematoxylin and Eosin staining, Mayer's Hematoxylin purchased from Electron Microscopy Sciences (26043-05) and Eosin was purchased from Acros Organics (61181-5000).

Antibodies:

The antibodies used in this experiment were listed below:

Anti-FAM83H rabbit polyclonal antibody was purchased from Sigma Aldrich (HPA024505). Anti-Kallikrein 4 polyclonal rabbit antibody (ab71234) was purchased from Abcam, INC. Anti-DSP(M-300) (sc-33587) was purchased from Santa Cruz Biotechnology, INC. Rabbit IgG (I-1000) was purchased from Vector Labs.

Sample collection and preparation:

Mice were euthanized using carbon dioxide gas, decapitated, skinned, and de-mineralized using 10% EDTA at pH 8. After 4-6 weeks, the decapitated heads were checked for proper demineralization and then gone through several ethanol washes starting with a 50% concentration then increasing the concentration to 80% and 90%, and then washing twice with the 100% ethanol. Each wash was for 20 minutes long and the samples were kept on a shaker during the washing period.

Afterwards, samples were left in xylene for 20 minutes and checked for clearness in the tissue color. Each sample was kept in paraffin wax overnight at 50-60 C°. On the following day, samples were chosen and embedded using paraffin wax with a mold on one side and a plastic holding box on the other side. The samples were then kept in a water bath overnight in the 4 °C.

On the following day, the samples were detached from the mold, then brought to the microtomes to produce 5-10 micrometer sections dispensed in a warm water bath and picked again using glass slides. The slides were kept for 1 hour in the oven at 50-60 °C to prevent any folds in the sections. Then, slides were numbered according to the first cut and dispensed into a water bath. Samples' cuts started from the medial line of

the head and directed outward and laterally to produce sagittal sections of the sample. Some modifications in the angulation were optimized to produce the desired orientation in each slide. Samples were picked from the warm water bath containing desired odontogenic structures valid for future processing and manipulation.

Immunohistochemistry (IHC):

Slides were de-paraffinized with xylene twice each for 5 minutes, followed by putting the samples through multiple ethanol washes in the following sequence: 100% ethanol twice each for 2 minutes, 80% ethanol for 2 minutes, 50% ethanol for 2 minutes and finally with distilled water for 2 minutes. Antigen retrieval was done using citric acid. This step took place by heating the citric acid between 80-90 °C in a microwave and afterwards the slides were dipped and left after to cool down to room temperature for 30 minutes. Slides were then picked and washed twice with PBS for 5 minutes. Blocking was carried out washing in PBS solution. Methanol (45ml) and hydrogen peroxides (5 ml) mix was used for a blocking step for 30 minutes. Samples were gone through washing in PBS twice for 5 minutes, then the slides were incubated with 100 microliters for each slide with 10% goat serum in PBS for a blocking step of the samples. This solution was left while the slides were covered with tin foil for 30

minutes at room temperature. Samples were gone through washing in PBS for 5 minutes twice. Sections were then treated with avidin for 20 minutes, then biotin for the same period of time after washing with PBS, shaded from light and at room temperature. After washing, slides were ready for primary antibody incubation depending on the desired dilution, antibodies were diluted in 5% normal goat serum in PBS and control sections were incubated with diluted normal rabbit immunoglobulin optimized to be at the same concentration as the primary antibody. Then the slides were left overnight in the cold room at 4 C°. Slides were then washed twice with PBS for 5 minutes and treated with biotinylated antibody as the secondary antibody, in a ratio of 2 microliters biotinylated antibody and 6 microliters of goat serum in 400 microliters of PBS, and sections were left 30 minutes at room temperature. The mixed reagents were used as 400 microliters of PBS, 8 microliters reagent, and 8 microliters reagent B. After washing the slides were stained with DAB solution with 2.5 milliliters distilled water, one drop of the buffering agent, one drop of hydrogen peroxidase, and two drops of DAB solution according to the manufacturer's recommendations. The DAB solution was removed from sections right after a brownish stain observed under the microscope. The reaction was deactivated using distilled water for 5

minutes. Time calculations were undertaken to achieve synchronization of the developing time for all sections of the same antibody. The primary antibodies tested were used with the following dilution:

KLK4 (1:250), DSPP (1:50), FAM83H (1:50),WDR72 (1:500) and AMELX (1:1000).

Hematoxylin and eosin (H&E) staining and hematoxylin counterstaining:

H&E staining was performed for randomly picked slides to show which section series carries the most appropriate tooth structure with enough of tooth structure to undergo the immunohistochemistry step.

Counterstaining took place using hematoxylin stain for no longer than 10 seconds, which was washed afterward under running distilled water then dipped into ammonium. After that slides dipped into eosin for 30 seconds, followed by a distilled water wash.

After this process, slides were subjected to gradual ethanol washes for dehydration starting with 50%, 80%, then 100% ethanol and then into

xylene twice. Each wash was two minutes long. Then, slides were mounted with paramount and a cover glass, and sealed. Slides were kept under weights to omit any possible bubbles and voids.

Observation and Photography:

Finally, slides were observed under electron microscopes and pictures at 10X power magnification were obtained using a Zeiss light microscope. Larger magnifications photography (20X, 40X) were captured using the light microscope of the imaging core facility of Boston University. All comparable sections underwent the same magnification processes and microscope settings to achieve conditions for maximum comparison.

Results:

The immunohistochemistry experiments were carried out and have shown different outcomes, according to each antibody and its specificity to variable odontogenic structures. Photos of each antibody treatment were compared to a control slide of a normal rabbit immunoglobulin(NRIG), as all antibodies used were rabbit derived.

Kallikrein 4 (KLK4):

We investigated the expression of Kallikrein 4 (KLK4) in adult mouse incisors. The results showed that immunoreactivity was observed in multiple locations in the pulp and in ameloblasts around the enamel on the tooth cross section shown in Figure 1 (A). In Figure 1 (B), (C) as indicated by the arrows in 20X magnification photo, the results showed a positive immunoreactivity in the ameloblasts in two different locations and also immunoreactive staining was observed in the pulp in figure 1 (D) (indicated by an arrow). On the other hand no immunoreactivity was identified on similar tooth locations when the NRIG was used (Figure 2 (A), (B) and (C)). Also, in unusual location for KLK4, the epithelial cells showed a faint brownish staining on the whole tooth section Figure 1 (A) as the arrow is indicating.

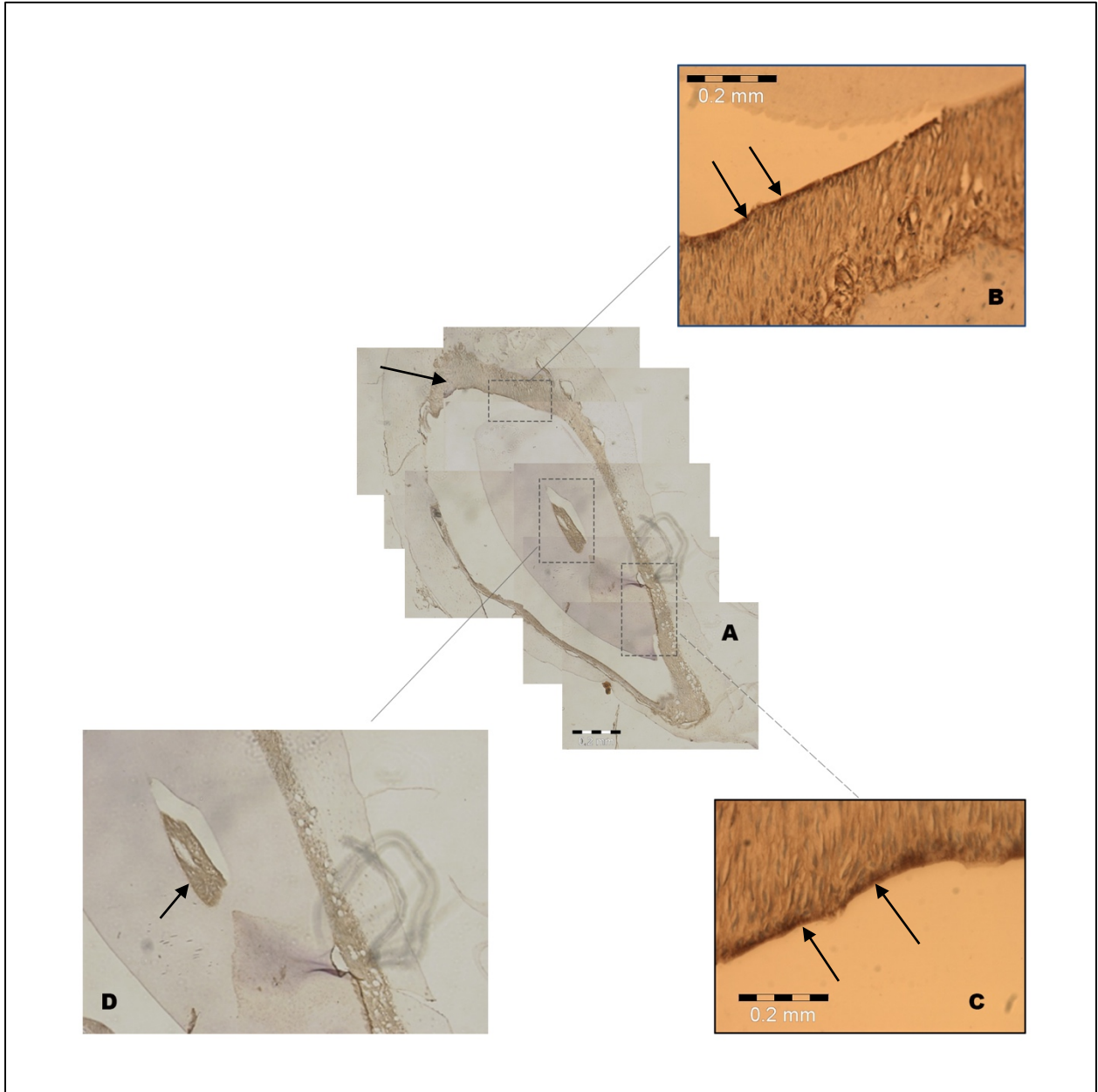


Figure 1. KLK4 1-250 concentration (experimental slide)

A. Whole tooth cross section.

B. 20X magnification.

C. 40X magnification.

D. 10X magnification.

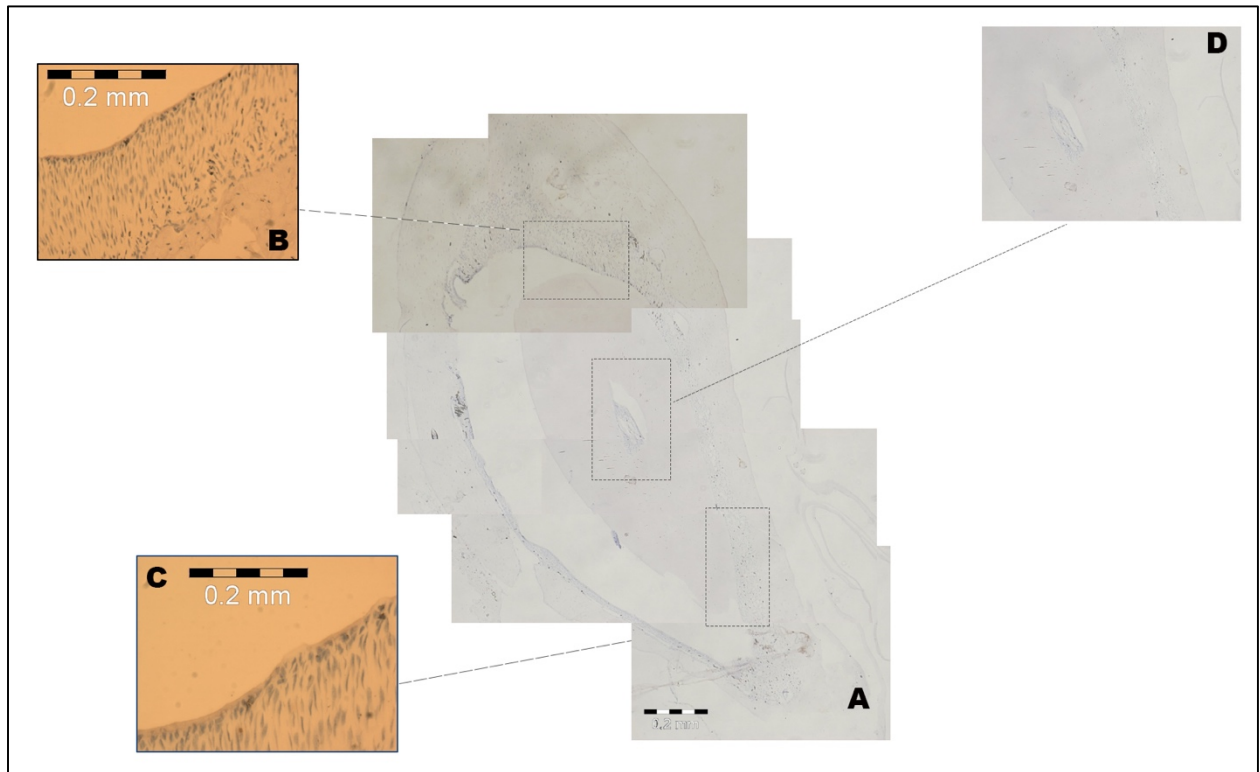


Figure 2. KLK4 NRIG (control slide).

A. Whole tooth cross section.

B. 20X magnification.

C. 40X magnification.

D. 10X magnification.

Dentin sialophosphoprotein (DSPP):

We investigated the expression of dentin sialophosphoprotein (DSPP) and the results showed immunoreactivity on experimental slide figure 3 (A) whole tooth cross section generally in the dentin hard structures, in the pulp and in the epithelial cells in one location of the section. In 10X magnified figure 3 (B) the arrows are pointing on the staining on the pulp tissues. In 20X magnification figure (C) as the arrow is indicating there is immunoreactivity identified through the dentin structure showing lines of stains along the odontoblastic processes. Also the experiment showed a stain on the epithelial cells in some area of the section. In figure 3 (D) there were stain observed at one location in the epithelial cells which attributed to non-specific stain. No immunoreactivity was observed when the (NRIG) was used in the control slide figure 4 (A), (B),(C),(D).

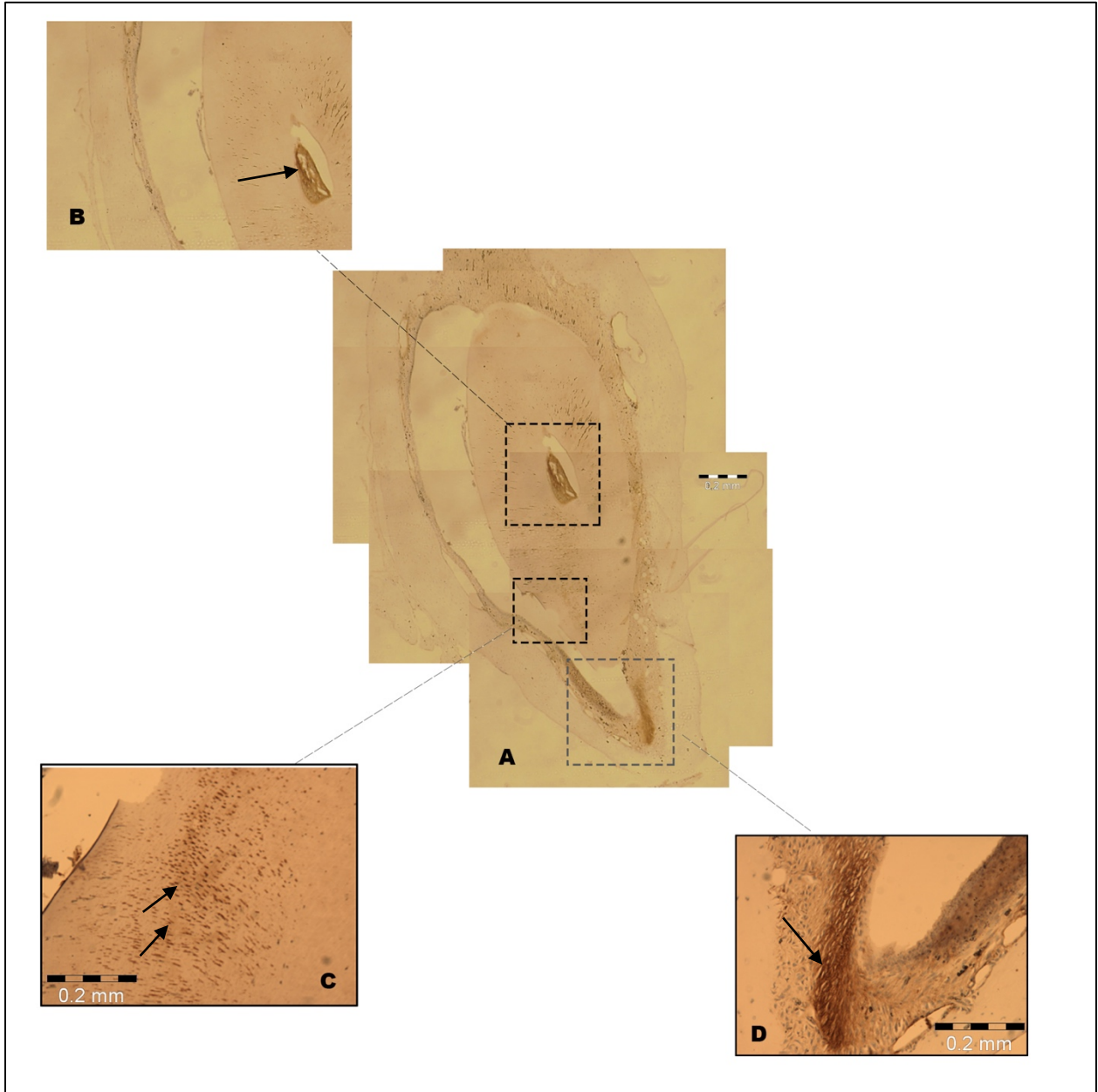


Figure 3. DSPP 1-50 (experimental slide)

A. Whole tooth cross section.

B. 10X magnification.

C. 20X magnification.

D. 20X magnification.

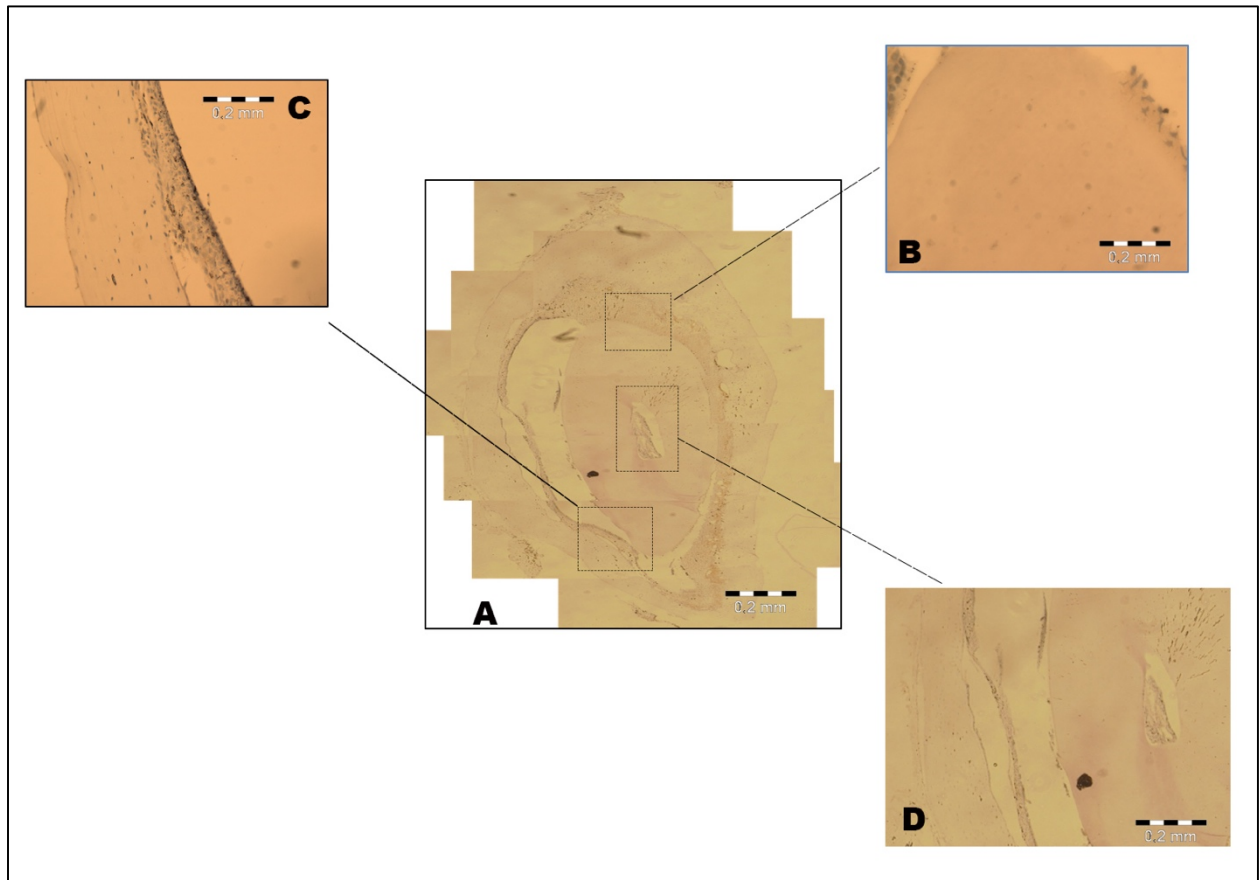


Figure 4. DSPP NRIG (control slide).

A. Whole tooth cross section.

B. 20X magnification.

C. 20X magnification.

D. 20X magnification.

Family with Sequence Similarities 83H(FAM83H):

Investigation of FAM83H expression was carried out using adult mouse incisors in figure 5 (A) and (B) the results showed a very faint immunoreactivity as the arrows are indicating on the ameloblasts in the experimental sections in figure 5 (B). No immunoreactivity was noticed when the NRIG was used in figure 6 (A), and B)

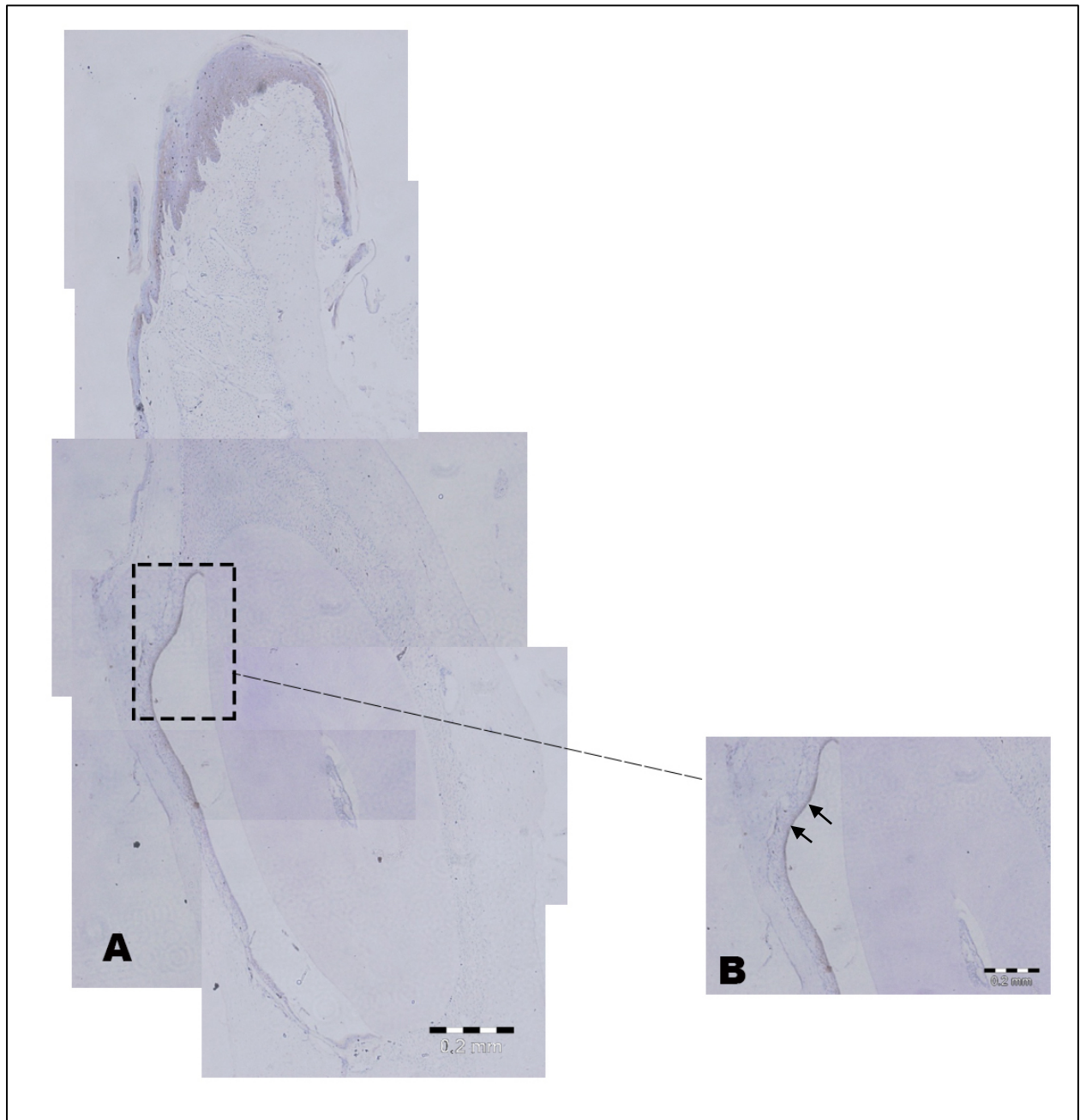


Figure 5. FAM83 1-50 concentration (experimental slide).
A. Whole tooth cross section.
B. 10X magnification.

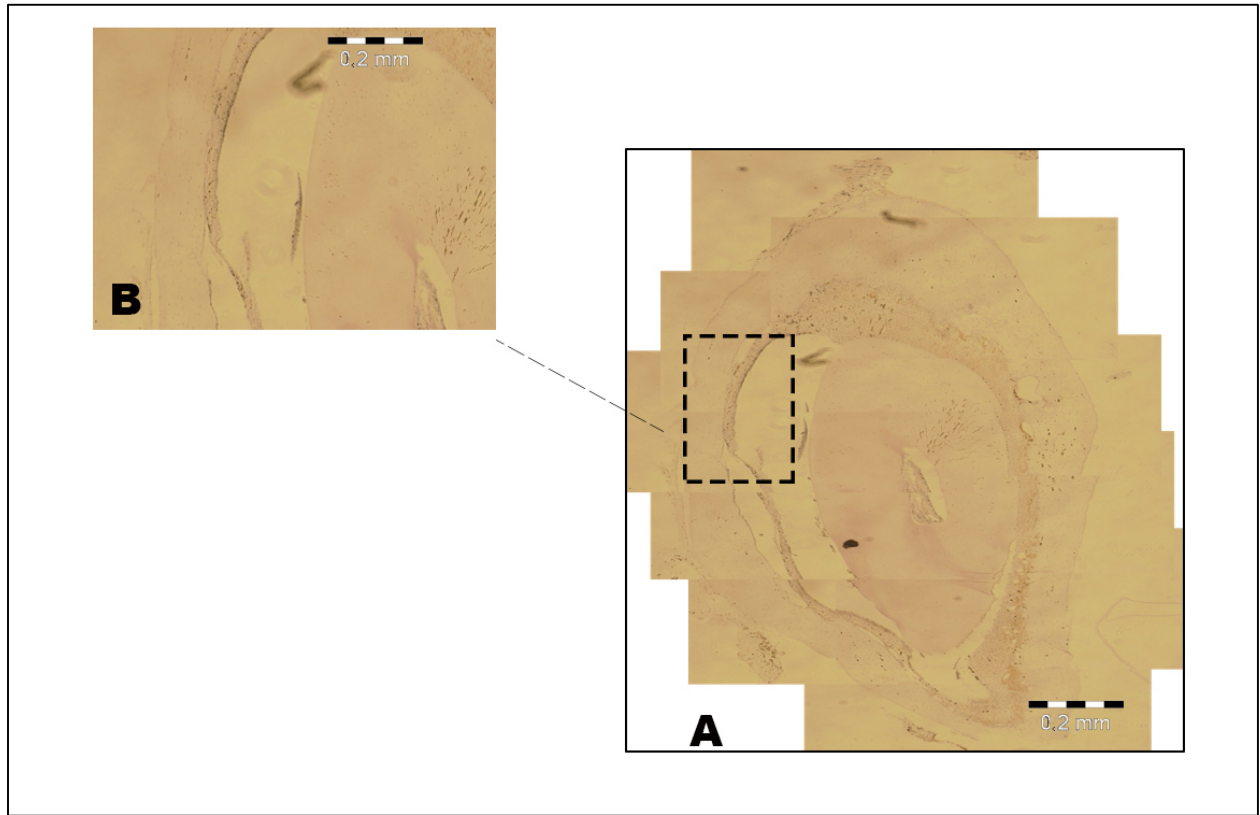


Figure 6. FAM83 NRIG (control slide).
A. Whole tooth cross section.
B. 10X magnification.

WD repeat-containing protein 72 (WDR72):

We investigated the the expression of WD repeat-containing protein 72 (WDR72) on adult mouse incisor figure 7 (A) and in (B) where the arrows are indicating and results showed weak immunoreactivity in the experimental slides in ameloblasts. No immunoreactivity was identified when NRIG was used in figure 8.

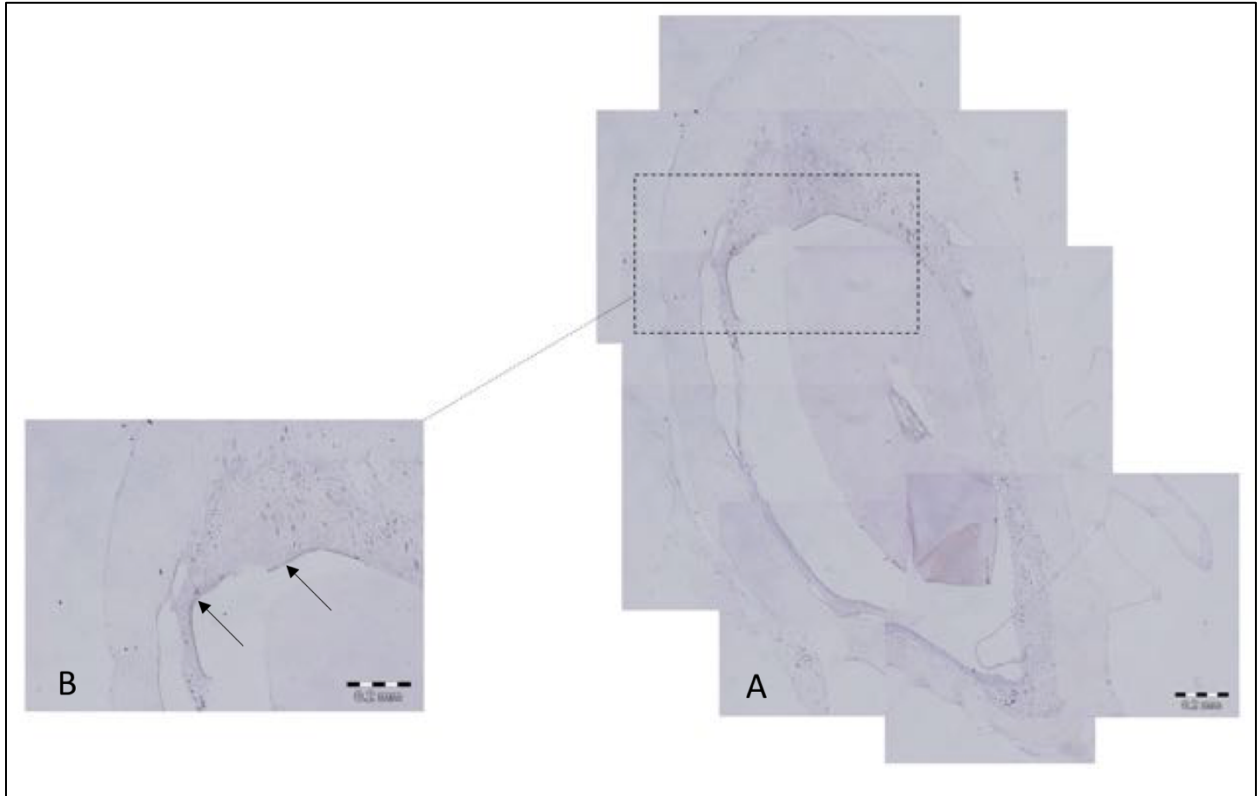


Figure 7. WDR72 1-500 (experimental slide).

A. Whole tooth magnification.

B. 10X magnification.

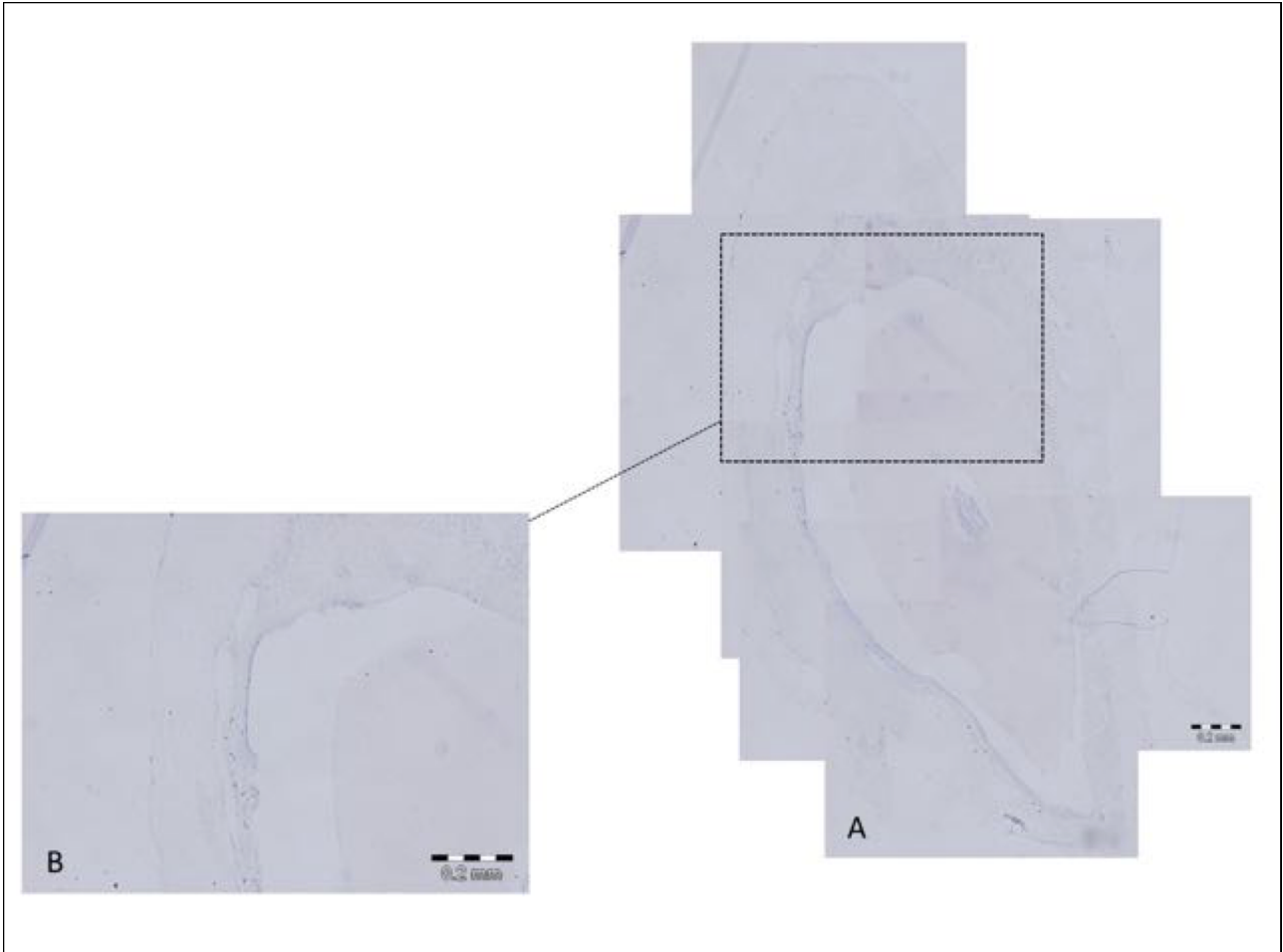


Figure 8. WDR72 NRIG (control slide).
A. Whole tooth cross section.
B. 10X magnification.

Amelogenin:

Investigation for the amelogenin antibody expression on was carried out using embryonic day 18.5 mouse incisor tissues. The results showed immunoreactivity was found in the enamel (Fig 9, indicated by straight arm arrows) and the ameloblasts (Fig 9, indicated by elbowed arm arrows). No immunoreactivity was identified when NRIG was used in (Figure 10).

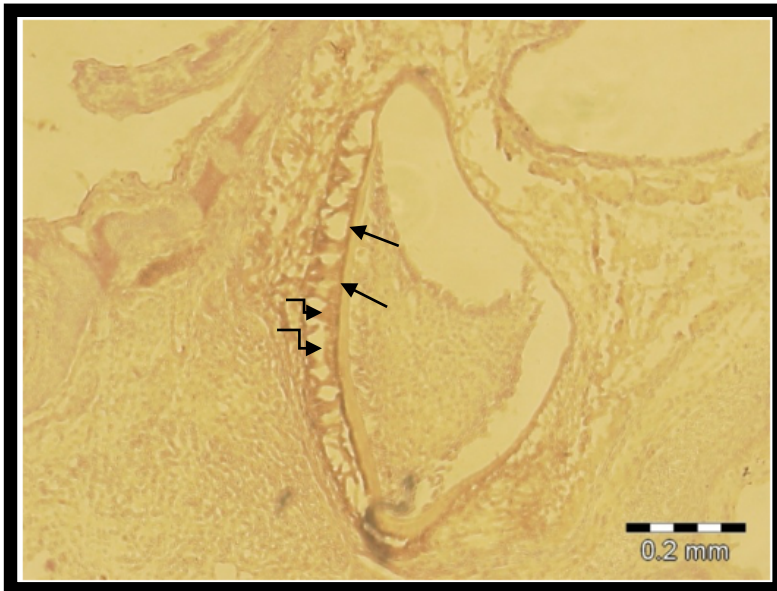


Figure 9. Amelogenin 1-1000 (experimental slide) 10X magnification.

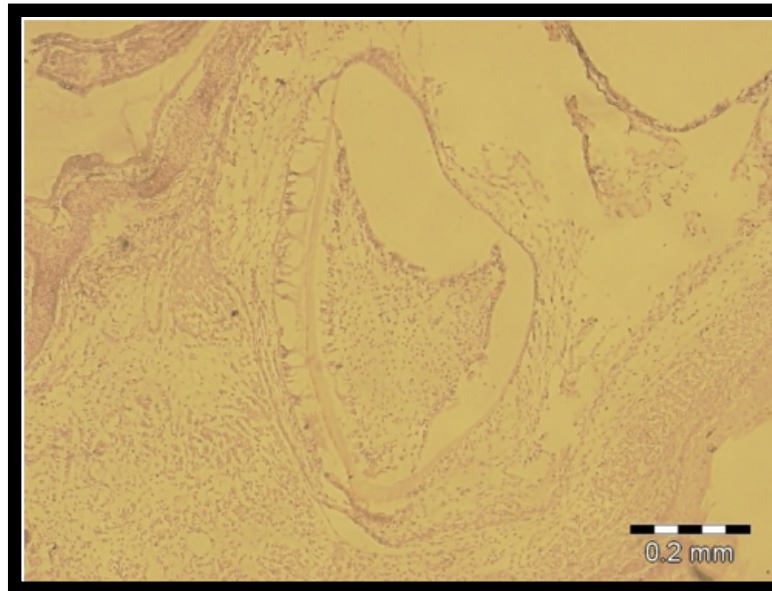


Figure 10. Amelogenin NRIG (control slide) 10X magnification.

Discussion:

The aim of the current study was to localize and evaluate the expression of the putative proteins responsible for the Amelogenesis Imperfecta and Dentinogenesis Imperfecta conditions. Current study utilized immunohistochemistry (IHC) to present the results, as it is a well-known methodology for locating proteins and testing their expression compared to a normal serum used as a negative control.

Enamel and dentin are two unique tissues that serve to build the odontogenic structures of the mouth. The inability of these two tissues to regenerate or even repair completely makes prevention of conditions that may sabotage the health of the resulting structures a vital subject for research. Enamel and dentin matrix proteins are major contributors in the physiological formation of teeth. Their expression and its intensity are variable around the tooth structure, as are their functions. On the other hand, the intensity of protein expression can be argued to be variable with regard to the time and stage of tooth formation.

According to Simmer et al (2009), localized expression against the KLK4 enamel protein was observed specially at the maturation stage around the

ameloblasts.³⁶ Our experimental results showed KLK4 protein expression in the ameloblasts around the enamel space. A high concentration of anti KLK4 antibody was used with the current method as the lower concentration did not show expression of the protein. On the other hand, the expression of KLK4 was found in the PDL and dental pulp, which has not been reported previously.

Previous studies have shown that DSPP is located in the odontoblasts and in the dentin matrix of wild type mice.³⁷ The DSPP immunoreactivity presented as strips that looked like the protein expression that was following the dentinal tubules path.³⁷ In our results the immunohistochemistry demonstrated similar results to the immunoreactivity in the dentin matrix, in the pulp and as lines crossing dentin thickness following the dentinal tubules path towards the pulp.³⁷

FAM83H was reported to have maximal expression at 14 days post natal mice in the ameloblasts, odontoblasts, and around the alveolar bone.

These results suggest that FAM83H may play an important role in the differentiation of ameloblasts and in enamel matrix calcification.³⁸ In the current study, little expression of FAM83H was detected in the ameloblasts, as shown in Figure 5, which agrees with previously mentioned studies. There was no expression in the bone or dentin. Also, it

has to be mentioned that the current study was performed on cross sectional slides that may not show the whole expression of the tooth.

A previous immunohistochemistry study to detect WDR72 localization on tissues showed similar results in regards to determining the location of the protein immunoreactivity in ameloblasts and enamel. This study demonstrated the expression of the WDR72 protein in the maturation and secretory stages. The maturation stage of tooth development showed the most prominent expression of WDR72.³⁹

Our results indicated similarities in the aspect of localization of the protein around the ameloblasts. In contrast, the current immunohistochemistry results did not show the prominent expression observed in the previously mentioned study. This can be attributed to the protocol used for IHC.

Moreover it may also be wise to hypothesize that the current experiment was carried out using cross sectional slides which may be missing antibody immunoreactivity towards apical or coronal directions if longitudinal section was used.

The amelogenin protein was studied at embryonic 18 day mice, and results showed the expression of amelogenin in the polarized ameloblast compared to the negative control.⁴⁰ In the present study, amelogenin was

detected in the ameloblasts and around the enamel matrix and enamel, compared to the control slides. The experimental slides showed elevated expression compared to the previous study that may be attributed to the concentration of the amelogenin antibody administered in our trail.

Conclusion:

The data presented here demonstrated the possibility of locating the proteins with important roles in enamel and dentin defects in mice teeth. These findings were supported by previous studies and conveyed the validity of IHC experiments in locating these proteins in odontogenic tissues. In addition, comparison of mice at different ages can be a point of interest in investigating the degree of intensity of protein expression in various age brackets.

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