On the dental enamel; formation, structure and growth tracks

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Oslo, 2012

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CONTENTS

INTRODUCTION

The present work has been carried out at the Department of Oral Biology, Faculty of Dentistry, University of Oslo, during the period 2010-2012. The work consists of two parts:

Part I: This chapter includes basic information concerning tooth development, enamel formation and structure, and growth tracks of dental enamel. It provides an overview of the development and biology of dental hard tissues, with emphasis on the dental enamel. The structure of mature enamel testifies to events that took place during enamel formation. The deposition of enamel is marked by the formation of growth lines, which reflect incremental growth. This topic is given special attention, as an introduction to the main part of this work, i.e. part II.

Part II: This part is presented as a manuscript entitled: "Incremental lines of mouse molar enamel". It deals with the geometry of the enamel growth tracks which is valuable tool for understanding temporal and spatial patterns in tooth morphogenesis. The growth tracks of mouse molar enamel have so far not been investigated and described. This work will be submitted to European Journal of Oral Sciences in January/February 2013.

I would like to extend an enormous debt of gratitude to my supervisor, associate professor Amer Sehic for giving me the opportunity to be part of his research group, for always being available for help, and for sharing with me his knowledge in tooth development and enamel structure. I am also deeply grateful to professor Steinar Risnes for his cooperation and contribution to the "part II" of this work.

My contribution to the part II:

- Study design
- Dissection and thoroughly cleaning of jaw segments (molars)
- Embedding in Epon
- Grinding (longitudinally), polishing and etching
- SEM microscopy
- Analysis and interpretation
- Writing of the paper *

* Writing of this paper would be impossible without help from Amer Sehic and Steinar Risnes. Their knowledge, experience and discussions were of the outmost importance for the completion of this article. At last, but not at least, their contribution and help with the writing of the paper were crucial for the high quality of this work.

Part I

TOOTH DEVELOPMENT

The first histological sign of tooth development is the appearance of a condensation of mesenchymal tissue and capillary networks beneath the presumptive dental epithelium of the primitive oral cavity. This process starts after 37 days of development where the mouth-epithelium starts to thicken in a continuous band shaped like a horseshoe extending around the mouth localized where the future upper and lower jaw will be situated. These two bands are called *primary epithelial bands* and they each evolve two important structures: the lingual situated *dental lamina* and the buccal situated *vestibular lamina* (Fig. 1). The dental lamina is the site from which teeth develop while the vestibular lamina becomes the cleft between the cheek and the tooth-bearing area.

The epithelial thickening expresses a host of signaling molecules that act to increase cell proliferation at the sites of tooth development. The proliferating epithelium grows further into the underlying neural crest-derived mesenchyme forming a bud. Condensation of the dental mesenchyme around the epithelial bud is followed by the induction of a signaling center at the tip of the epithelial bud, the enamel knot. The enamel knot was first described almost 100 years ago, and it is histologically visible as a bump in the center of the inner enamel epithelium at the cap stage. This knot of epithelial cells expresses key signaling molecules, such as *Shh*, *Fgf4*, *Bmp4* and *Wnt10b*, and has been considered as an important signaling center for tooth development and tooth morphogenesis. It has been shown that the enamel knots are transient structures, their disappearance by the end of the cap stage being *Bmp4*-dependent. It has been suggested that the primary enamel knot contributes to the secondary enamel knots. However, this theory is unclear, with fate-mapping experiments and proliferation studies providing both positive and negative evidence. The folding of the inner enamel epithelium at the bell stage, possibly associated with the secondary enamel knots, results in the formation of a complex multi-cuspid tooth. The deposition of dentin and enamel

extends from the epithelium-mesenchymal interface, the outline of which in turn defines the tooth shape. Further, molars develop tertiary enamel knots next to the enamel-free areas at the cusp tips. The size and shape of the primary enamel knot seems to be a clue for the generation of the exact degree of curvature of the oral epithelium.

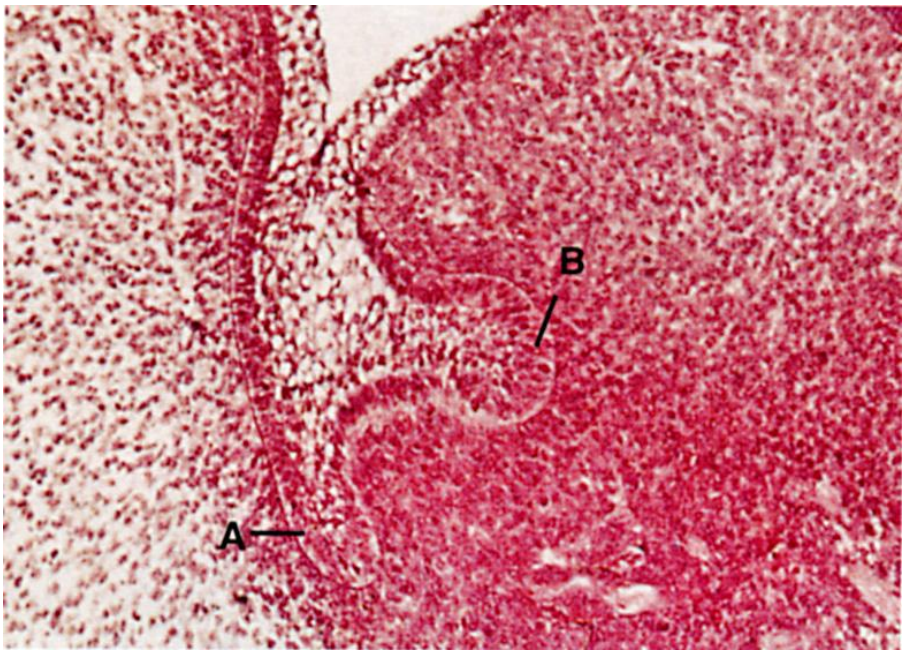

Fig.1. The vestibular lamina (A) and the dental lamina (B) seen at the 7th week of intra uterine life. From Oral anatomy, histology and embryology, fourth edition.

Tooth development can be divided into three overlapping stages: *initiation, morphogenesis* and *histogenesis*. The process is regulated by interactions between mesenchymal and epithelial tissues. The initiation is the phase when the localization of the future teeth in the jaw is determined. Invaginations appear in the dental lamina with the presence of tooth germs. These invaginations will be the sites where the deciduous teeth will evolve. Every site is surrounded by the mesenchymal tissue. During the morphogenesis the shape of the tooth is

determined by cell-proliferation and cell-movement. In this phase, tooth development can morphologically be divided into bud, cap and bell stages.

Bud stage:

In this stage of tooth development the enamel organ is undifferentiated, and is characterized by an invagination of the dental lamina which grows into the underlying ectomesenchyme. Mesenchymal cells are tightly packed, they support the epithelial bud and continue proliferating around the bud as the epithelial cells also proliferate. This process is called ectomesenchyme condensation. In the further differentiation from bud to cap stage the tooth type is determined by expression of growth factors like Bmp-4 and Msx-1 from the condensating mesenchymal cells. This is an example of how the epithelial tissue and the mesenchymal tissue cooperate in evolving the teeth and why the differentiation would never succeed if one of them were absent.

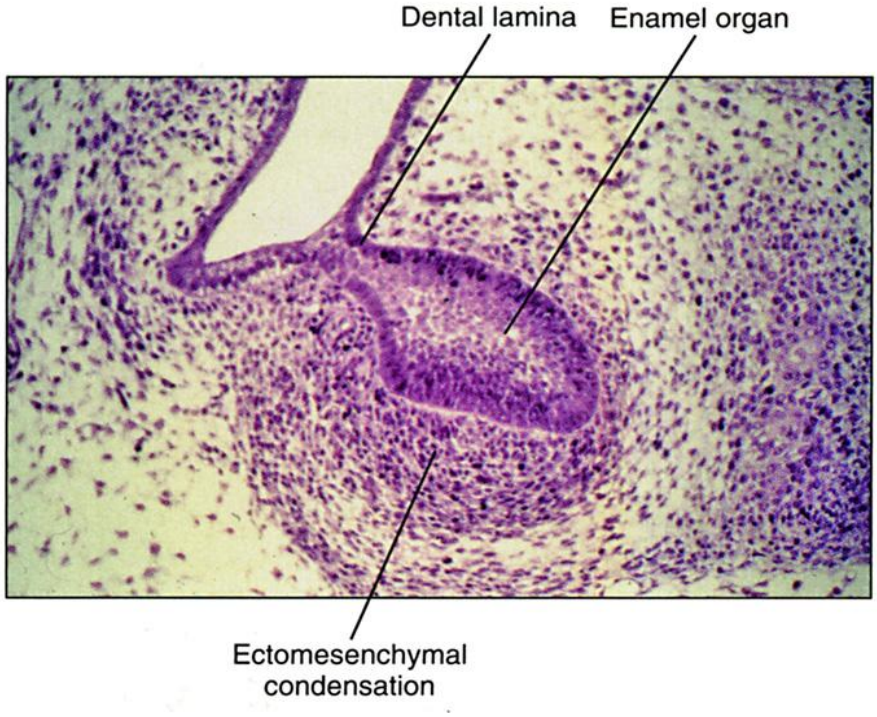

Fig. 2. Early cap stage of tooth development. A condensation of the ectomesenchyme associated with the epithelial cap is identified easily. From Ten Cate`s Oral Histology, eighth edition.

Cap stage

The tooth bud continues to grow, and the enamel organ and the surrounding tissues are easier to identify. This stage is easily identified because of its particular shape. The enamel organ shaped like a cap resting on the supporting mesenchyme tissue has given the stage its name. The condensed ectomesenchyme cells situated under the cap looking like they are forming a ball are called the dental papilla (Fig. 3), which will evolve to dentin and pulp of the tooth. The rest of the mesenchyme tissue around the enamel organ and the dental papilla is called dental follicle and will be the precursor to all the supporting tissue of the tooth (Fig. 3). Now it is possible to see the differentiation of the epithelial cells in the enamel organ were the cells rounded in the middle together with the more peripheral cells will form the external and internal enamel epithelium. The internal layer of epithelial cells has a columnar shape and consists of more RNA and enzymes while the external layer keep a cuboidal shape. The cells in the middle of the enamel organ are linked together by desmosomes.

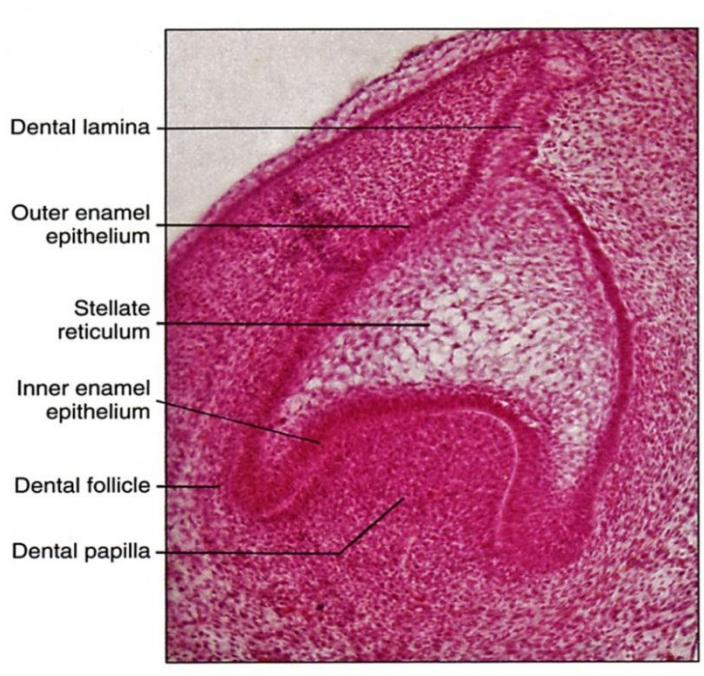

Fig. 3. Beginning of cellular differentiation within the enamel organ. Central cells form the stellate reticulum. The peripheral epithelial cells are differentiating into the inner and outer enamel epithelium. From Ten Cate`s Oral Histology, eighth edition.

By the late cap stage the cells produce a lot of glycosaminglycans which they excrete into the intercellular room; this stimulates influx of fluid resulting in the larger mass of intercellular space. The epithelial cells are drawn apart but are still held together by the desmosomes. This is the beginning of the development of stellate reticulum which will not be finished until the late bell stage. The name comes from the resemblance that the epithelial cells get, because of being drawn but still attached to desmosomes, giving them the star-shape look. The enamel organ is the precursor to the tooth enamel. In the late cap stage and early bell stage the tooth is proceeding into the next step in differentiation: histogenesis.

Bell stage

In this stage of tooth development the tooth germ is bell-shaped, giving it this name. This stage consists of the overlapping morphogenesis and histogenesis where during the former the inner layer of epithelial cells which are columnar determine the shape of the crown of the tooth. Later, ameloblasts and odontoblasts start to acquire their real phenotype. The enamel organ starts to exhibit more histodifferentiation by developing four distinct layers: the external enamel epithelium, stellate reticulum, stratum intermedium and the internal enamel epithelium (Fig. 4 & 5). The external and internal layers of epithelium are continuous and "meet" at the cervical loop were the cell division continues until the tooth is completely developed. Stratum intermedium evolves from the cells originating from the stellate reticulum and the inner enamel epithelium and their function is mainly to produce enzymes. During this stage the dental lamina ruptures, the contact with the oral epithelium is absent, and the dental lamina between the tooth germs disappears.

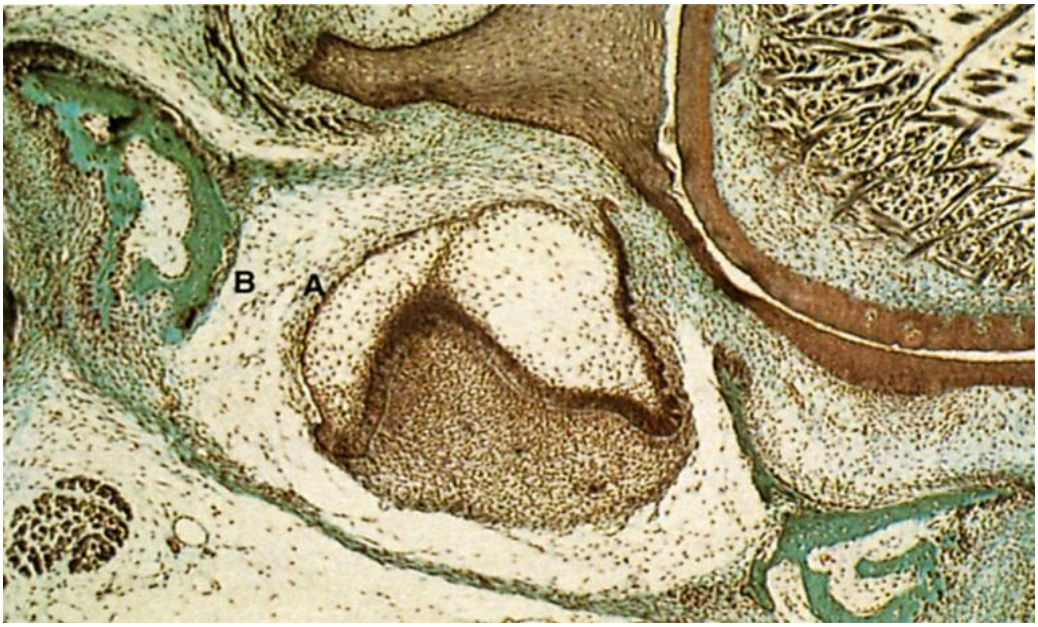

Fig. 4. Early bell stage of tooth development. A = inner investing layer of dental follicle, B = outer layer of dental follicle. From Oral anatomy, histology and embryology, fourth edition.

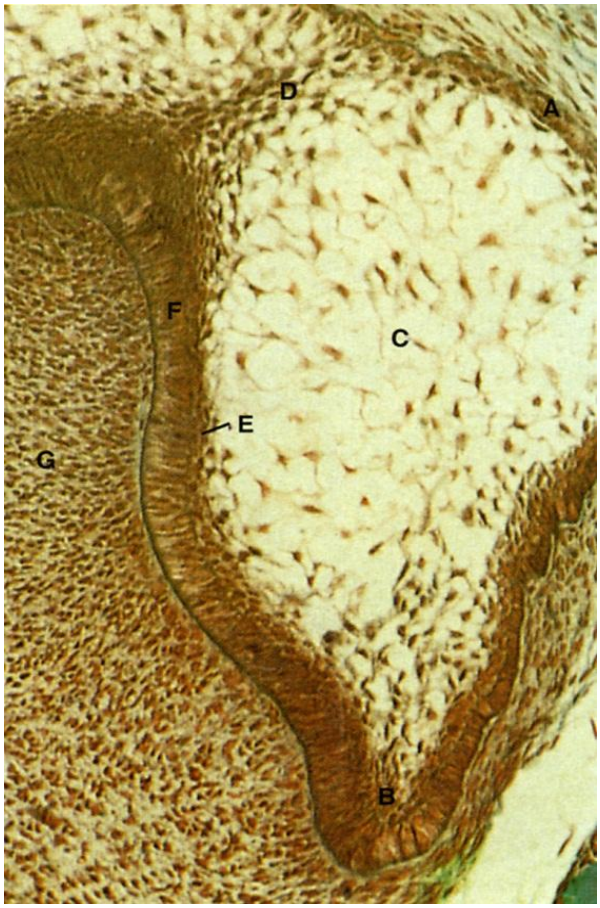

Fig. 5. A high-power view of early bell stage of tooth development. A = external enamel epithelium, B = cervical loop, C = stellate reticulum, D = enamel cord, E = stratum intermedium, F = internal enamel epithelium, G = dental papilla. From Oral anatomy, histology and embryology, fourth edition.

In the late bell stage, an important structure develops, i.e. the enamel knot which consists of a cluster of cells in the center of internal enamel epithelium forming a bulge into the dental papilla. Its function is mainly signalizing, and interestingly it does not have a proliferative function. It signalizes molecules like BMP, fibroblast growth factors and transcription factors like Shh. At this stage the nerve fibers and blood vessels are present in the dental papilla and they participate in tooth development, especially after beginning of dentinogenesis.

The next step in tooth development is production of hard tissue. This occurs by differentiation of ameloblasts and odontoblasts. Odontoblasts are differentiated from ectomesenchymal cells in the dental papilla space under the inner layer of enamel epithelium. These undifferentiated ectomesenchymal cells start to grow larger in size rapidly and ultimately differentiate into dentin producing cells; odontoblasts. Odontoblasts start to produce dentin matrix which eventually mineralizes. During this process they move towards the center of the dental papilla. Simultaneously, the inner enamel epithelium starts differentiating into ameloblasts. It has been proven that signals from both sides are crucial for differentiation of both odontoblasts and ameloblasts. Before the first dentin layer is produced the enamel organ receives blood from vessels in the dental papilla. After the formation of the first dentin layer this source is cut off and the inner enamel epithelium has no blood supply. However, this is resolved by the nourishment that is supplied when the stellate reticulum is dissolved. At this time the ameloblasts are almost differentiated and therefore not in the need of blood supply and will start the process of producing enamel; amelogenesis.

ENAMEL FORMATION AND STRUCTURE

Enamel is the only epithelially derived calcified tissue in mammals and its structure is unique. The formation of mineralized enamel matrix is a result of expression of tissue-specific genes in differentiated ameloblasts. Ameloblasts secrete two major classes of proteins: glycosylated and non-glycosylated. The non-glycosylated proteins are the hydrophobic amelogenins, constituting about 90% of proteins in the enamel matrix. Amelogenins are believed to function as the principal organizer of enamel deposition, but lately they have been suggested to be involved also in root formation, periodontium regeneration and to function as growth factors. The glycosylated or non-amelogenin enamel proteins include tuftelin, ameloblastin and enamelin. The first non-amelogenin protein characterized was tuftelin, but its function in tooth development is still not well understood. Tuftelin has also been found to be expressed in other organs such as kidney, lung, liver and testis. Ameloblastin represents about 5% of the enamel matrix and has been immunolocalized in the secretory ameloblasts and in the entire thickness of the enamel matrix. Studies carried out on the interaction between ameloblastin and amelogenin suggest a co-operative function in the scaffolding needed for formation of enamel. Mouse recombinant ameloblastin acts as a growth factor increasing cell attachment and proliferation of periodontal ligament cells *in vitro*. Ameloblastin has also been found in pre-odontoblasts, pulpal mesenchymal cells and Hertwig's epithelial root sheath cells, but its function in these tissues as well as in ameloblasts is still not fully understood. Enamelin is a large enamel matrix protein that has also been immunolocalized in the secretory ameloblasts and in the developing enamel matrix. *Enamelin* is thought to be the main candidate gene responsible for the autosomal-inherited form of amelogenesis imperfecta (AI), while mutations in *amelogenin* lead to X-linked AI.

Tooth enamel is unique among mineralized tissues because of its high mineral content. Enamel is made up of highly organized, tightly packed crystallites, hydroxyapatite,

$Ca_{10}(PO_4)_6(OH)_{2,}$ that compromise 87 percent of its volume and 95 percent of its weight. Whereas other mineralized tissues are about 20 percent organic material, mature enamel has less than 1 percent organic matter. Enamel crystallites contain more than one thousand times the volume of corresponding crystals in bone, dentin and cementum. Enamel crystals are extremely long relative to their thickness and highly oriented. They generally extend from the underlying dentin toward the surface of the tooth and are organized into bundles, called prisms. Superior organization and mineralization give dental enamel its outstanding physical properties, making it the hardest tissue in the vertebrate body.

It is easy to deduce that dental enamel formation is under genetic control. The process of enamel formation, or amelogenesis, occurs predictably in tooth after tooth, generation after generation. The size, shape, shade, and even caries susceptibility of dental enamel can be passed from parent to offspring. Genetic diseases are associated with enamel malformations that range from total enamel agenesis to localized defects. Therefore, the formation of dental enamel is somehow encoded in our genes, of DNA. But how can a gene encode a mineral? The answer is that in can`t, at least not directly. DNA can only encode RNA, and most of the RNA it encodes is used to make proteins. Dental enamel formation is highly specialized, and the proteins most directly involved in enamel biomineralization are specific for it. As a consequence, defects in the genes encoding enamel proteins generally cause enamel malformations without affecting other parts of the body. There are, however, numerous genetic syndromes associated with dental defects of all types.

Amelogenesis occurs in stages in a well-delineated extracellular compartment. It is the process when the enamel is produced and mineralized. It can be divided into five stages: presecretory stage, secretory stage, transition stage, maturation stage and postmaturation stage.

Presecretory stage consists of differentiation of ameloblasts and resorption of the basal lamina (Fig. 6). Ameloblasts which initially are cuboidal shaped differentiate into columnar cells over 60 µm in length and 2-4 µm in width. They undergo a polarization were important cell organs like the nucleus and mitochondria are placed in the bottom of the cell close to the stratum intermedium (Fig. 6). At this stage the cell is called *preameloblast*.

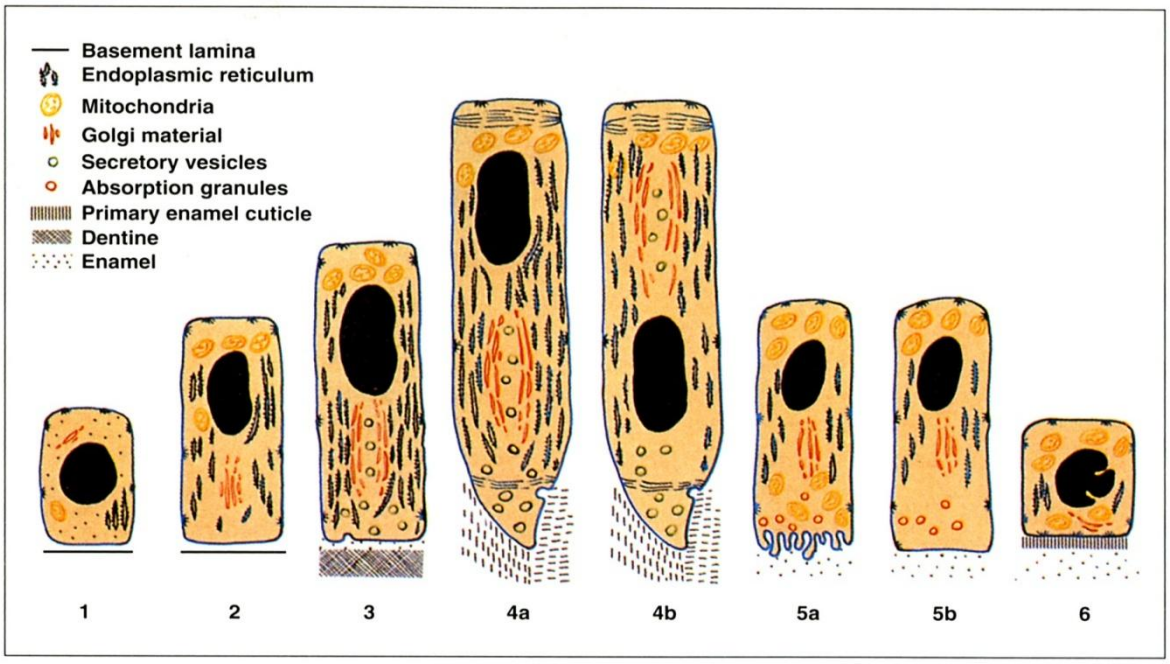

Fig. 6. The life cycle of an ameloblast. The cells of inner enamel epithelium (1) start to differentiate at the future cusp tip. The differentiating cell becomes columnar (2) and the nucleus moves to the part of the cell furthest from the dentine. The cell starts to secrete the initial enamel matrix (3), and as it retreats the Tomes process develops (4a). In this phase two appearances of the cell can be distinguished by the position of the nuclei within the cell: high (4a) and low (4b). When the full thickness of enamel has formed, ameloblasts lose the secretory extension, the Tomes process (5a). During the maturation phase there is a regular, repetitive modulation of cell morphology between a ruffled (5a) and a smooth (5b) surface apposed to the enamel.Once the maturation is complete, the cell regress in height (6). From Oral anatomy, histology and embryology, fourth edition.

Initially, a basal membrane separates the preameloblasts from the dental papilla and odontoblats. This marks the future enamel-dentin junction. Odontoblasts are

differentiated under signalization from the internal enamel epithelium which is the precursor for ameloblasts. These signals which are dominated by TGF family are deposited in this basal membrane and on to the mesenchyme cells that are differentiating to odontoblasts. When the first layer of dentin is formed enzymes are exuded from the dental papilla and the basal membrane is broken down. Under a short time after basal lamina degradation ameloblasts and odontoblasts are in intimate contact and can exchange signals. The odontoblats are the first to lay their dentin matrix and this is the signal which stimulates ameloblasts to start secretion. The ameloblast starts secreting enamel matrix which later will become crystallized. This first layer will be aprismatic because of the absence of Tomes process which has yet not been differentiated.

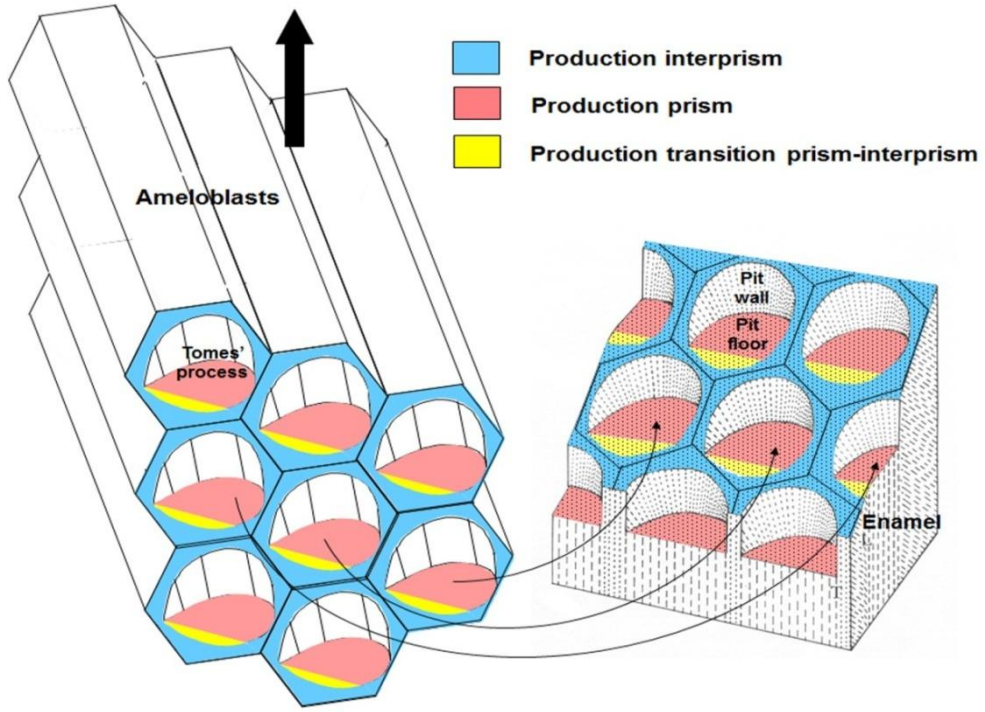

Fig. 7. Schematic representation of a block of developing enamel, and ameloblasts that are removed from enamel surface. The enamel is characterized by impressions on the developing enamel surface. Tomes`processes occupy pits in enamel surface. Enamel matrix is produced from two different locations from the ameloblasts; 1) From the rim at the base of the Tomes`processes resulting in interprism, and 2) From the flat aspect of Tomes`processes related to the pit floor resulting in prisms. From Steinar Risnes.

The secretory stage marks the full production of enamel matrix from the beginning until it's finished. The mineralization also starts during this phase. In this stage the Tomes process is differentiated at the end of the columnar cell of the ameloblast which now is longer (Fig. 6). This site is also the secretory part of the cell. When the enamel matrix is secreted from the ameloblast they are pushed away from the dentin junction. It is indeed the shape of the Tomes process that determines the prismatic look of the enamel (Fig. 7). There are three types of pattern which the prims are built by (Fig. 8). It's mainly the size of the ameloblast that determines which pattern it will shape.

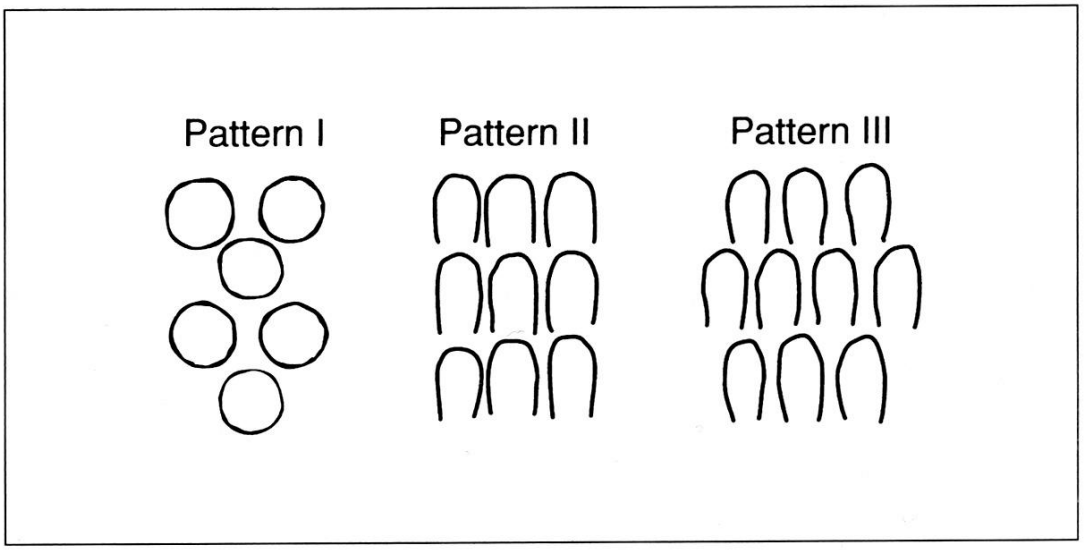

Fig.8. The three prism patterns seen in human enamel. In pattern I enamel the prisms are circular. In pattern II enamel the prisms are aligned in parallel rows. In pattern III enamel the prisms are arranged in staggered rows such that the tail of a prism lies between two heads in the next row, giving a keyhole appearance. From Oral anatomy, histology and embryology, fourth edition.

The crystal molecules start to elongate in a thin line and the more enamel matures the thicker they get. The structural richness of dental enamel is related to the spatial arrangement of the hydroxyapatite crystals. These are not arranged at random; in mammals,

the crystals are organized into a pattern of prisms (rods) and interprismatic (interrod) substance (Fig. 7 & 9). Basically, the prisms are discrete, rod-like entities running from the dentine to the enamel surface, while the interprismatic substance constitutes a continuum in between the prisms (Fig. 9). The hydroxyapatite crystals are differently oriented in prisms and interprismatic substance, and this is the sole basis for a distinction between the two; in the prisms, the crystals are oriented with their long axis roughly parallel with the long axis of the prism, while in the interprismatic substance, the crystals tend to be oriented perpendicular to the incremental lines (Retzius lines). At the end of this stage the ameloblasts eventually lose their Tomes`process and produce a thin, superficial prism-free enamel.

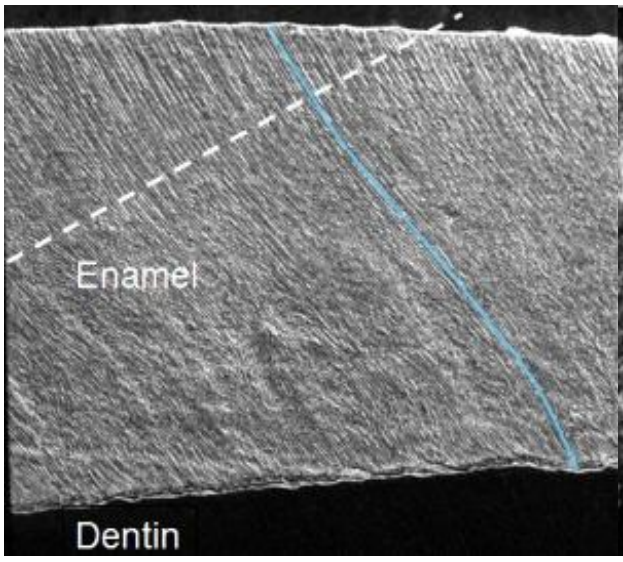

Fig. 9a. Scanning electron microscopy image of human enamel, showed in longitudinal section. The blue line indicates a continous prism from enamel-dentin junction to the enamel surface. From Steinar Risnes.

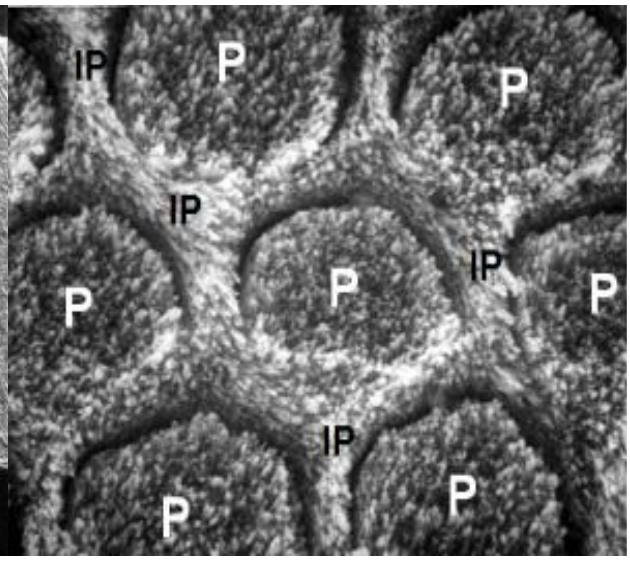

Fig. 9b. Scanning electron microscopy image of higher magnification of enamel in a section indicated by white line in A, showing prisms (P) and interprism (IP). From Steinar Risnes.

During the transition stage ameloblasts change form and function for the new required qualities. The first enamel is now complete but has a content of 65% water, 20% organic material like proteins and only 15% inorganic hydroxyapatite and is therefore very

porous. A maturation process is ahead to make the hard final enamel. This process is also carried out by ameloblasts. They have to undergo changes to be able to contribute to the maturation; this takes place through the transition stage. Ameloblast height is reduced and even the number of cells decrease to 50% by apoptosis. The cell organelles, which produced the matrix, now disappear by autophagocytosis.

In the maturation stage of amelogenesis the enamel proteins are degraded and further mineralization occurs. Ameloblasts secrete calcium, phosphate and carbonate ions into the matrix, and remove water and degraded proteins the matrix. Enamel crystallites increase in width and thickness at the expense of intercrystallite space. During this stage the protein removal and mineralization is complete. At the end, in the postmaturation stage, the enamel organ finally retires and is followed by eruption of the tooth and exposure to oral environment.

GROWTH TRACKS IN DENTAL ENAMEL

All hard tissue in the body like bone, cementum, dentine and enamel grow in layers. If the growth was continues one would not expect any layers, but if the growth is periodic, like in these cases, then one will expect to see a boundary between these layers. These boundaries have a common name: *growth tracks* or *lines*.

The ameloblasts move when they produce enamel. This movement brings them from enamel-dentine junction to the surface of the enamel. Although each single ameloblast makes an individual contribution to enamel production, the layered building of enamel is a joint venture of a continuous sheet of ameloblasts, the ameloblastema. The movement of ameloblastema as a whole is mirrored by the incremental lines of enamel, the Retzius lines (Fig. 10), while the path pursued by each individual ameloblast is traced out by the prisms. The fine, horizontal grooves on the surface of the crown, the perikymata grooves, represent the external manifestations of the Retzius lines (Fig. 11).

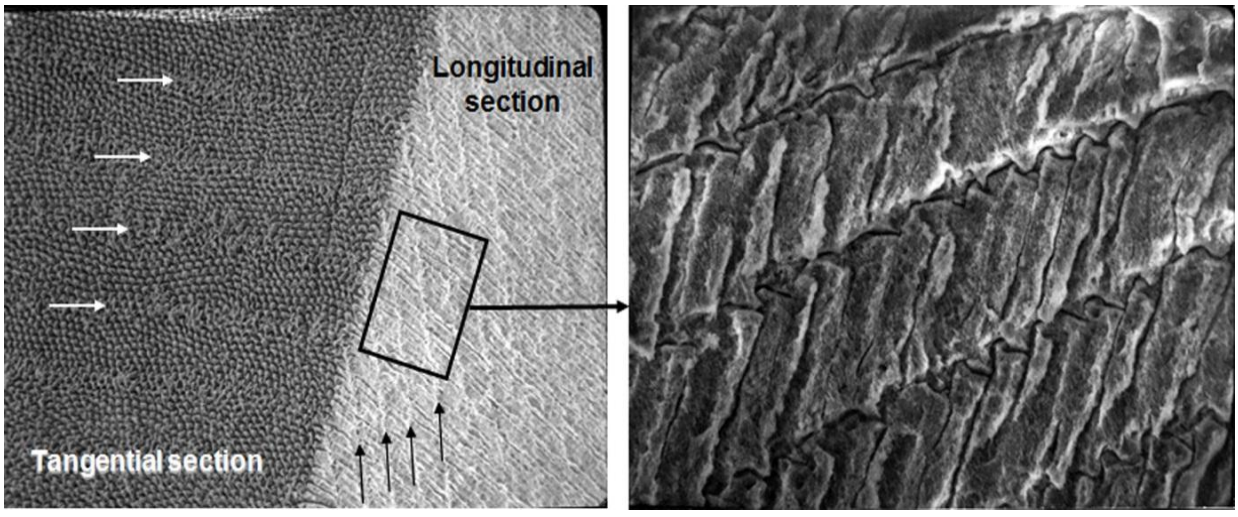

Fig. 10. Scanning electron microscope images of human enamel exhibiting Retzius line. Shows region where two ground planes meet. Retzius lines in outer enamel (arrows) are continuous across edge where tangential and longitudinal planes meet. From Steinar Risnes.

The Retzius lines and the prism cross-striations are important structural features related to the growth of enamel. The Retzius lines represent two-dimensional cuts of three-dimensional growth planes, and the basis for their visibility is only partly understood. The regularly spaced Retzius lines prominently present in outer and cervical enamel are thought to represent a 6-10 day rhythm in enamel production. They are structurally characterized by a discontinuity cleft, at least in their outer part, and an increase in interprism at the expense of prisms. The prism cross-striations are thought to represent a daily rhythm in enamel formation. Their visibility has been ascribed to periodic variations in composition along the prisms. Dark bands with reduced concentration of crystals have been observed after acid etching. The cross-striations have been linked to another periodic structural feature occurring along the prisms, the prism varicosities/undulations, but this association has been questioned. Both Retzius lines and cross-striations tend to be accentuated by acid and caries.

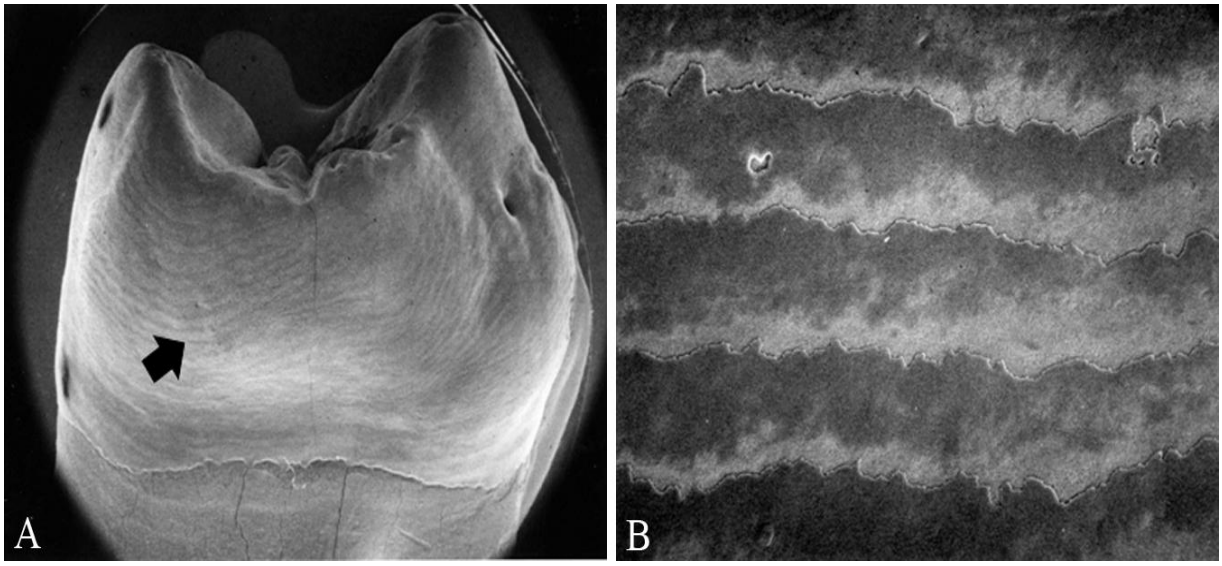

Fig. 11. Scanning electron microscope images of human premolar (A) and external enamel (B) showing the horizontal grooves on the surface of the crown, the perikymata grooves, which represent the external manifestations of the Retzius lines. B) Higher magnification of external enamel at the position indicated by black arrow in A. From Steinar Risnes.

One fundamental property of incremental lines would be that they are continuous in nature, i.e. that the Retzius lines represent one-dimensional cuts of two-dimensional, curved growth planes within three-dimensional enamel cap. This has been implicitly accepted based on indirect evidence, such as their characteristic outline in longitudinal and transverse ground sections, the fact that both fluoride-induced and tetracycline-induced lines mimic this characteristic outline, and that their width as observed in light microscope varies with the angle of observation. Later, a direct morphological proof has been provided where Retzius lines in adjoining planes cut through the enamel are shown to be continuous across the edge where the two planes meet (Fig. 10).

The continuous nature of the Retzius lines means that at the moment during enamel production when a certain growth line/plane is introduced, the whole ameloblastema is involved. Regularly spaced Retzius lines, which indicate a specific rhythm in enamel apposition, could either be caused by an inherent and synchronized rhythm in the activity of the secreting ameloblasts, or by a more general rhythm which also affects ameloblastema. The accentuated Retzius lines, often referred to as pathologic, are probably caused by systemic influences which may range from physiologic fluctuations, as in formation of the neonatal line, to pathologic conditions. This is corroborated by the fact that the pattern of accentuated Retzius lines in teeth within the same dentition is very similar in the enamel that was produced at the same time.

So far, Retzius lines have not been conspicuous and observed in rat and mouse enamel, possibly because enamel apposition occurs at a much faster rate than in human enamel. The following part of this work (part II) is concerned with the occurrence and periodicity of incremental markings through the enamel thickness of mouse molars, which so far have not been systematically studied and described.

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Part II

Incremental lines in mouse molar enamel

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A running title: Growth tracks in mouse molar enamel

Abstract

The purpose of the present study was to investigate the occurrence and periodicity of enamel incremental lines in mouse molars in an attempt to draw attention to some key questions about the rhythm in the activity of the secreting ameloblasts during formation of mouse molar enamel. Incremental lines generally appeared as grooves of variable distinctness, and were only observed cervically, in the region about 50-250 μm from the enamel-cementum junction. The lines were most readily observable in the outer enamel and in the superficial prism-free layer, and were difficult to identify in the deeper parts of enamel, i.e. in the inner enamel with prism decussation. However, in areas where the enamel tended to be hypomineralized the incremental lines were observed as clearly continuous from outer into inner enamel. The incremental lines in mouse molar enamel exhibited an average periodicity of about 4 μm, and the distance between the lines decreased towards the enamel surface. Taken together, our results show that incremental lines are visible in mouse molar enamel, and that they probably represent daily rhythm in enamel formation. This study witnesses the layered apposition of mouse molar enamel and support the theory that circadian clock probably regulates enamel development.

Keywords: Dental enamel; Molar; Mouse; Scanning electron microscopy; Ameloblasts

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Introduction

Circadian clock genes are expressed in mouse molars, suggesting that these genes may be involved in the regulation of ameloblast and odontoblast functions, such as enamel and dentin matrix secretion and mineralization (1). Using an ameloblast cell line, other researchers have explored the potential links between circadian control and stage-specific regulation of ameloblast genes (2). Another recent study has demonstrated that circadian clock genes in an ameloblast cell line and amelogenin gene (*Amelx*) in 2-day postnatal mouse molars oscillate in a circadian pattern (3). These studies suggest that circadian clock genes modulate enamel development, and that amelogenesis is subject to diurnal rhythms in gene-expression levels and cell activity during development of mouse molar enamel.

Enamel formation starts at the enamel-dentin junction and proceeds outward by layered apposition of the matrix produced and secreted by the retreating ameloblasts. This movement of ameloblasts brings them from the enamel-dentin junction to the surface of the enamel. The path pursued by each individual ameloblast is traced out by the prisms, while the movement of the ameloblast layer as a whole is mirrored by the incremental lines of enamel, the Retzius lines (4). These lines, therefore, indicate the position of the ameloblast layer and of the developing enamel surface at different points of time and may evidence physiological or pathological events affecting enamel formation. A system of evenly spaced incremental lines, especially evident in the outer and cervical enamel of humans and other primates has been thoroughly described, and is highly indicative of a physiological rhythm in enamel formation (5-9). Shorter increments, the prism cross-striations, probably represent a diurnal rhythm in enamel formation (10-12). The number of prism cross-striations between any two regularly spaced Retzius lines is reported to vary between 6 and 11 (9). These incremental growth tracks, Retzius lines and prism cross-striations, represent an internal record of time

which, linked to a time scale, may serve as a valuable tool in determining the rate of enamel formation (11) and the crown formation time (13).

Rodent and human enamel exhibits the same basic structural elements, prisms and interprism. However, the spatial arrangement of the prisms, i.e. the prism pattern, is considerably different. In rat and mouse incisors the enamel exhibits two main layers, an inner layer with extreme prism decussation and an outer layer with parallel and incisally inclined prisms (14;15). Rat and mouse molar enamel resembles incisor enamel, but prism decussation is absent in some areas (16;17). Incremental lines (Retzius lines and prism cross-striations) are not conspicuous in rat and mouse enamel (14;15;18), possibly because enamel apposition occurs at a faster rate than in human enamel (14;19). Lines resembling incremental lines, with a periodicity of about 1 μm, have been observed in the aprismatic enamel in mouse molars (16). Korvenkontio reported the presence of incremental lines in certain rodent species, but with no conclusive evidence for rat and mouse enamel (20). Also, lines which are parallel with the surface of developing rat enamel have been induced experimentally by injections of sodium fluoride (21) and tetracycline (22).

The occurrence and periodicity of incremental markings in mouse molars have not been thoroughly investigated. We have occasionally observed lines resembling incremental lines in mouse molar enamel. In view of the renewed interest in clock genes and circadian rhythms in amelogenesis we wanted to do a systematic study on the occurrence, conspicuousness and periodicity of incremental lines in mouse molar enamel.

Materials and methods

Experimental animals

Ten phenotypical adult mice (CD-1 strain) were randomly selected for the study. All animals were kept at a 12h light:dark cycle at 21^{o}C with a relative humidity of 65%. Prior to experimental use, animals were given standard laboratory fodder and water *ad libitum*. The animals were kept according to the regulations of the Norwegian Gene Technology Act of 1994.

Scanning electron microscopy

After cervical dislocation, the right upper and lower jaws containing all three molar teeth were dissected out and fixed in 70% ethanol. After fixation, all soft tissue was carefully removed by dissection under a stereomicroscope and by light brushing under running tap water. The specimens were air-dried, embedded in Epon, and ground longitudinally in a mesiodistal direction under a stereo-microscope using grits 800 and 1200 3M (3M, St. Paul, MN, USA) waterproof silicone carbide paper in a specially designed apparatus (23). The ground surface was then polished against the backside of the 3M waterproof silicone carbide paper with 0.05 µm particle size alumina powder (Buehler Micropolish, Buehler, Lake Bluff, IL, USA) in water. After careful brushing under running tap water, teeth were etched for 45 s in 0.1 % nitric acid, air-dried overnight, sputter-coated with 30 nm gold-palladium and observed in a Philips XL30 ESEM (Philips, FEI, Netherlands) operated at 10 kV.

Measurements and statistical analysis

SEM micrographs of ground and etched molars were used to investigate the occurrence of incremental lines and also their periodicity, i.e. the distance between the lines. Mean values and standard deviations were calculated using Microsoft Excel Worksheet (Microsoft Office Excel, 2010).

Results

Occurrence of incremental lines in mouse molar enamel

The enamel exhibited the characteristic division into two main layers, i.e. outer enamel and inner enamel (Fig. 1a). Incremental lines generally appeared as grooves of variable distinctness (Fig. 1-3). The lines were only observed cervically, in the region about 50-250 µm from the enamel-cementum junction. They were visible in 67-74% and 61-78% of maxillary and mandibular molars, respectively (Table 1). The lines were most readily observable in the outer enamel and in the superficial prism-free layer, and were difficult to identify in the deeper parts of enamel, i.e. in the inner enamel with prism decussation (Fig. 1). However, in areas where the enamel tended to be hypomineralized (darker with less distinct crystals and prisms/interprism in SEM) the incremental lines were observed as clearly continuous from outer into inner enamel, where their visibility faded more or less abruptly or gradually (Fig. 2 & 3). Where the lines were not unduly blurred by the hypomineralized state, it appeared that crystal discontinuity and/or crystal deficiency may contribute to their visibility (Figs. 1b, 2c,d). Closest to the enamel-cementum junction the incremental lines tended to be parallel with the enamel surface (Figs. 1a,b, 2a,b, 3a,b), further occlusally they reached the surface at an angle of about 10-15° (Figs. 2c,d, 3c,d).

Periodicity of incremental lines in mouse molar enamel

There was no significant difference in periodicity between distal and mesial aspect of the molars. Therefore, the results are presented collectively for both aspects of the teeth (Table 1). The incremental lines in mouse molar enamel exhibited an average periodicity of about 4 µm (Table 1). The distance between the lines decreased towards the enamel surface (Fig. 1-3). Close to the enamel surface the periodicity of incremental lines was between 1.0 – 2.1 µm,

while deeper within enamel the distance between the lines was between 4.4 – 5.1 µm (Table 1). However, the distance between the incremental lines close to enamel surface, i.e. in the outer enamel and in the superficial prism-free layer, was somewhat variable. Closest to the enamel-cementum junction the periodicity of the lines was smaller (Fig. 2b, 3b) compared with periodicity of the lines further occlusally (Fig. 2d, 3d).

Discussion

The main clock genes known to control circadian rhythms and related functions in many tissues, *Bmal1*, *Clock*, *Per1* and *Per2*, are expressed in mouse molars (1). Interestingly, it has also been shown that the amelogenin gene (*Amelx*) in 2-day postnatal mouse molars oscillate in a circadian pattern (3), supporting the assumption that amelogenesis is subject to diurnal rhythms in gene-expression levels and cell activity controlled by clock genes. A rhythmic production of enamel could be expected to show up as incremental lines in the mature enamel, comparable to Retzius lines and/or prism cross-striations in human enamel. The lack of reports on incremental lines in mouse enamel is may be due to lack of interest, lack of adequate methods and lack of conspicuousness of such lines. The present study provides novel evidence for the presence of incremental lines in mouse molar enamel which may be linked to clock genes and circadian rhythms in the expression of major genes associated with amelogenesis.

Retzius lines and prism cross-striations are structural features of dental enamel that are closely related to the formation and growth of dental enamel. Regularly spaced Retzius lines in human enamel have a periodicity of about 7-11 days (9), while prism cross-striations are believed to represent a daily rhythm in enamel formation (10-12). Incremental lines of enamel have been extensively used to investigate the chronology of crown formation in living and fossil mammals (9;24). It has been suggested that daily incremental lines are related to

differences in the chemical composition and porosity of the inorganic phase of enamel during the circadian cycle (25). Incremental lines in mammalian enamel can be observed both by light microscopy (5-6) and electron microscopy, especially after acid etching (7-9, 26). As observed in this study, incremental lines were visible in the cervical enamel of about 70% of mouse maxillary and mandibular molars. The lines were most readily observable in the outer enamel and in the superficial prism-free layer. This is in accordance with earlier findings in human enamel showing that Retzius lines in the superficial enamel are more readily exposed after acid etching than Retzius lines in the deeper parts of enamel (26). Interestingly, when observing ground sections of mouse molars in the light microscope, no conclusive incremental lines were identified that with high reliability could be distinguished from scratches resulting from grinding. This is in contrast to previous findings where light microscopy of a ground section of a human permanent tooth exhibited more Retzius lines than SEM of the same section (9). It has been suggested that Retzius lines may owe their visibility to different types of structural and/or compositional characteristics or to different degrees of expression of such characteristics (8). In our study the incremental lines were difficult to identify in the deeper parts of enamel, except in areas where the enamel tended to be hypomineralized. It is reasonable to believe that the structural and/or compositional characteristics of hypomineralized enamel constitute or contribute to line identity and visibility. Maybe the difference between crystals (number, size, discontinuities) within and between the lines is greater in hypomineralized than in fully mineralized enamel. Also, the extensive secretory surface along one aspect of the elongated Tomes' process of mouse ameloblasts would, presumably, result in jagged and indistinct incremental lines, and even more so considering the inclination of Tomes' processes in opposite directions in adjacent transverse rows of ameloblasts when producing the extreme prism decussation. Therefore, it is more surprising than not that in some instances incremental lines seem to be continued into

the inner enamel with prism decussation (Figs. 2,3). An explanation may be that lines primarily are associated with interprism formation sites at the base of the Tomes' processes and from the remaining secretory surface after recession of the Tomes' processes, which both are secretory sites that jointly, for all secretory ameloblasts at that stage, form lines parallel with the ameloblastema. The crystals associated with the incremental lines observed in the outer enamel and superficial enamel are oriented transversely to the lines and clearly belong to the interprism system of crystals.

Our results showed that incremental lines in mouse molar enamel exhibited an average periodicity of about 4 µm. However, this is based on measurements restricted to the cervical enamel. The appositional secretion rate of ameloblasts in mouse mandibular incisor enamel has been estimated to about 6 µm/day (14). There was a tendency for the periodicity of the incremental lines observed in mouse molars to increase in a direction toward the cusp and toward the enamel surface. The same phenomenon has been observed for prism cross-striations in human enamal. It may, therefore, be speculated that an average incremental line periodicity higher than 4 µm in mouse molars would have been obtained if there had been measurable lines throughout the whole extent of the enamel.

In conclusion, it seems reasonable to assume that the observed lines in mouse molars are incremental lines and that they represent a diurnal rhythm in enamel formation. Furthermore, we think that the lines observed in mouse enamel are associated with the interprism/aprismatic system of crystals.

The enamel on the mouse mandibular first molar forms in about 10 days. In order for the observed incremental lines to be interpreted as morphological manifestations of a circadian rhythm in enamel appositional growth, an extrapolation of the system of observed lines throughout the enamel should result in a total number of about 10 lines, We tried this and, discarding the cervical lines that do not reach the cemento-enamel junction (Figs. 2a, 3a),

and assuming an increased periodicity in occlusal and dentinal direction, i.e. reaching 6-7 μm, we arrived at about 10-15 lines in total. Which, considering the uncertainty of the available data, is an indication that the observed lines represent morphological manifestations of a diurnal rhythm in mouse enamel formation.

Acknowledgements

The expert technical assistance of Mr. S. Ø. Stølen and Mr. B. Geronimo is gratefully acknowledged.

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Table 1. Occurrence and periodicity of incremental lines in mouse molar enamel.

Shows percentage of molars exhibiting incremental lines. Mean values and standard deviations of the periodicity of incremental lines are also shown, with range of periodicity in parentheses. Experimental details are otherwise given in Materials and methods.

	Maxillary molars	Mandibular molars
First molar	67% 3.9 ± 1.3 µm (1.2 - 4.8)	78% 3.8 ± 1.5 µm (1.0 - 5.1)
Second molar	73% 4.0 ± 1.2 µm (1.7 - 4.4)	68% 4.1 ± 1.2 µm (1.9 - 4.9)
Third molar	74% 4.2 ± 1.3 µm (2.1 - 5.0)	61% 4.2 ± 1.4 µm (2.0 - 5.1)

Legends to Figures

Fig. 1. SEM images of enamel from mesial aspect of first maxillary molar (M1 sup), about 50 µm from enamel-cementum junction. The enamel exhibits the characteristic division into two main layers, i.e. outer enamel and inner enamel. The black arrows in panel b indicate position and direction of incremental lines in outer enamel. The periodicity of incremental lines tends to decrease towards enamel surface. D = dentin, R = resin, OE = outer enamel, IE = inner enamel, p = prism, ip = interprism. The bar represents 10 µm in panel a, and 5 µm in panel b.

Fig. 2. SEM images of enamel from distal aspect of second mandibular molar (M2 inf). b-d) Higher magnification of enamel at the positions indicated by black arrow-heads in panel a. The enamel appears hypomineralized (darker with less distinct crystals and prisms/interprism). The black arrows indicate position and direction of incremental lines in enamel. Incremental lines are seen to proceed some distance into the inner enamel. The periodicity of incremental lines tends to decrease towards to enamel surface. D = dentin, R = resin, OE = outer enamel, IE = inner enamel. The bar represents 50 µm in panel a, and 10 µm in panels b, c and d.

Fig. 3. SEM images of enamel from distal aspect of third mandibular molar (M3 inf). b-d) Higher magnification of enamel at the positions indicated by black arrow-heads in panel a. The enamel appears hypomineralized (darker with less distinct crystals and prisms/interprism). The black arrows indicate position and direction of incremental lines in enamel. Although incremental lines are only barely visible in the inner enamel, there remains an impression of such lines throughout the enamel thickness, especially in panel c. The periodicity of incremental lines tends to decrease towards to enamel surface. D = dentin, R = resin, OE = outer enamel, IE = inner enamel. The bar represents 25 µm in panel a, and 10 µm in panel b, c and d.

Figure 1

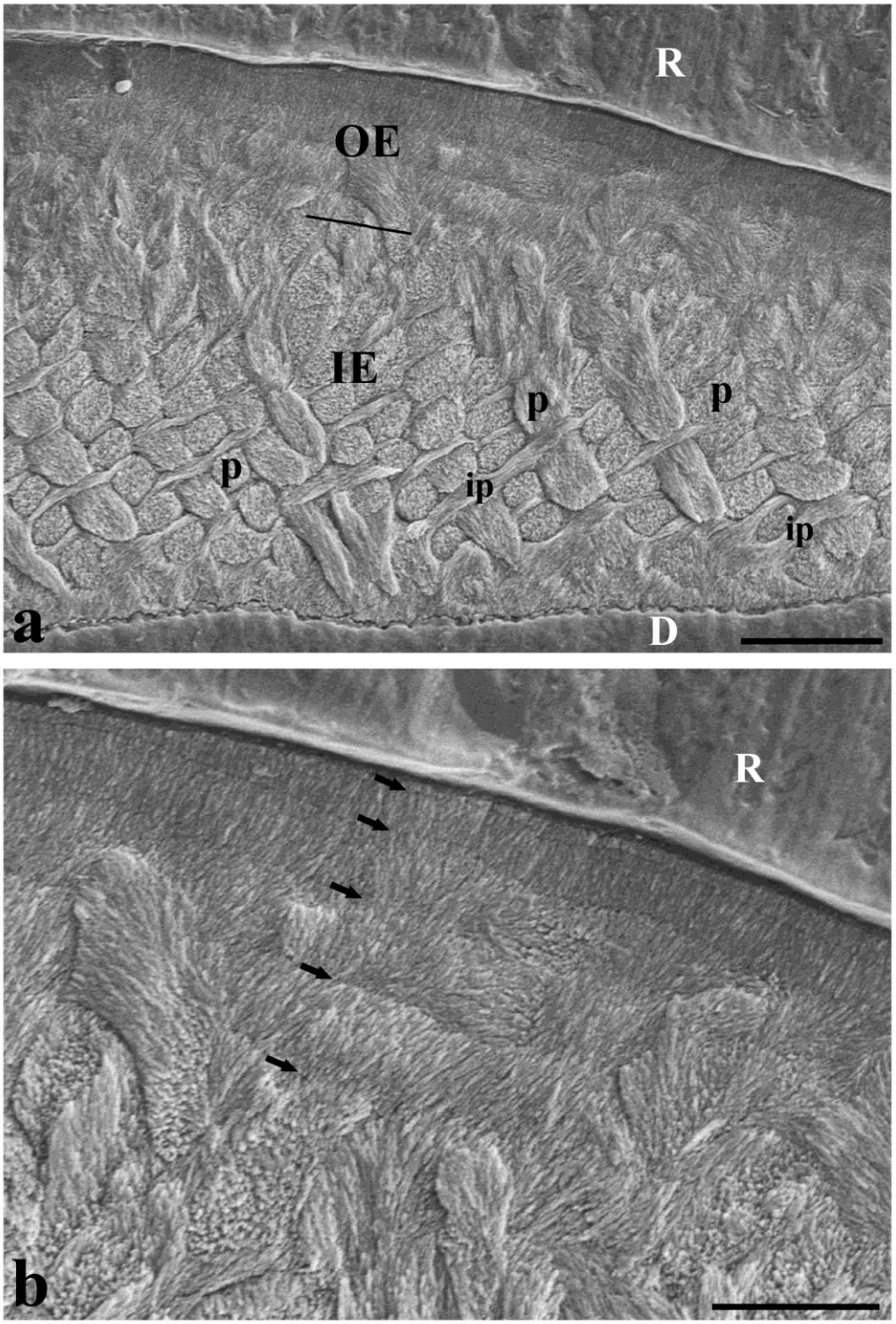

Figure 2

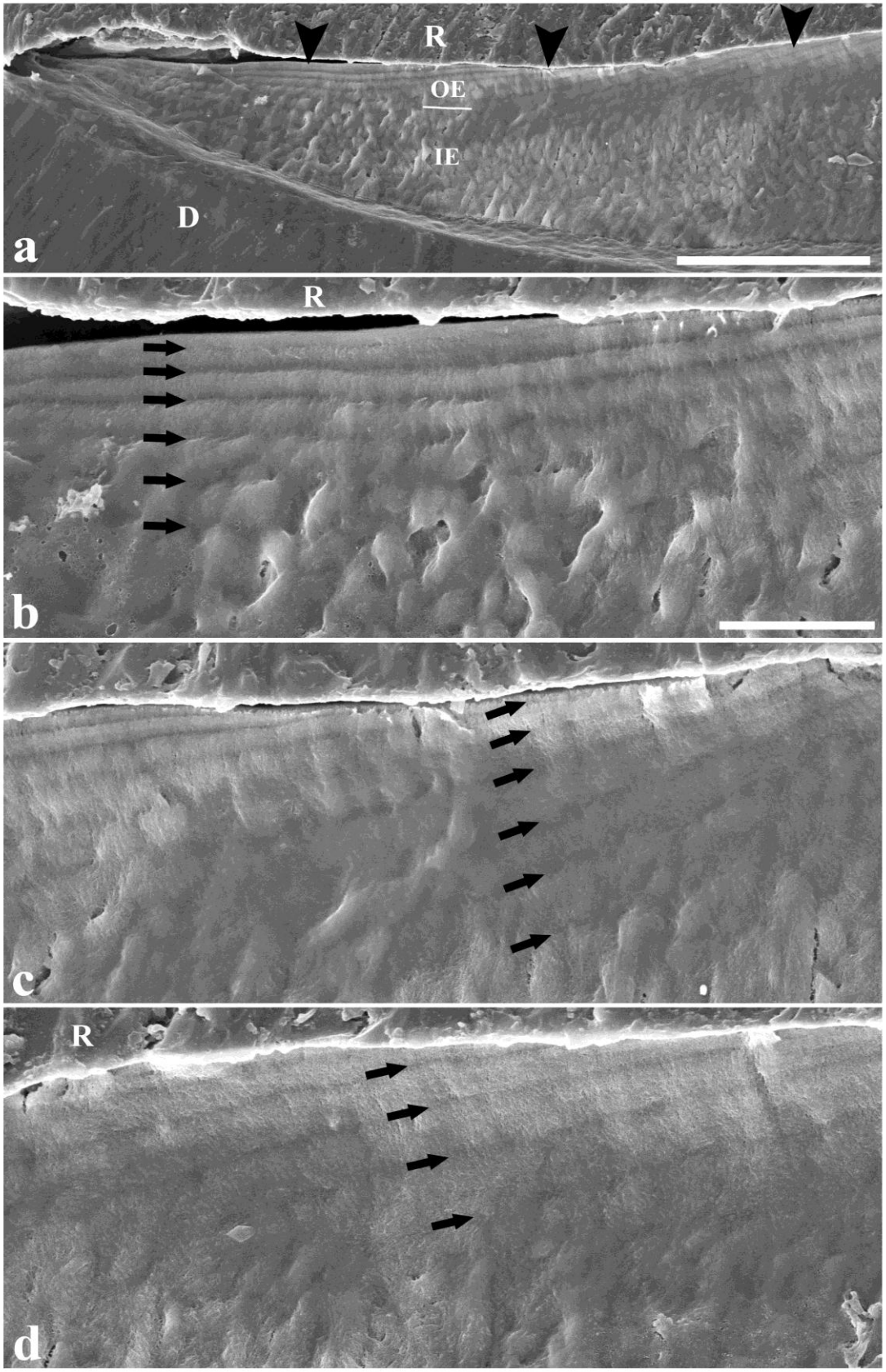

Figure 3