Dentin: Structure, Composition and Mineralization

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1. ABSTRACT

We review firstly the specificities of the different types of dentin present in mammalian teeth. The outer layers include the mantle dentin, the Tomes’ granular and the hyaline Hopewell-Smith’s layers. Circumpulpal dentin forming the bulk of the tooth, comprises intertubular and peritubular dentin. In addition to physiological primary and secondary dentin formation, reactionary dentin is produced in response to pathological events. Secondly, we evaluate the role of odontoblasts in dentin formation, their implication in the synthesis and secretion of type I collagen fibrils and non-collagenous molecules. Thirdly, we study the composition and functions of dentin extracellular...
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Table 1. Global composition of dentin

<table>
<thead>
<tr>
<th>Component</th>
<th>Weight (in weight)</th>
<th>Volume (in vol)</th>
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<tr>
<td>Mineral phase</td>
<td>70%</td>
<td>40-45%</td>
</tr>
<tr>
<td>Organic matrix</td>
<td>20%</td>
<td>30%</td>
</tr>
<tr>
<td>Water</td>
<td>10%</td>
<td>20-25%</td>
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matrix (ECM) molecules implicated in dentinogenesis. As structural proteins they are mineralization promoters or inhibitors. They are also signaling molecules. Three different forms of dentinogenesis are identified: i) matrix vesicles are implicated in early dentin formation, ii) collagen and some proteoglycans are involved in the formation of predentin, further transformed into intertubular dentin, iii) the distal secretion of some non-collagenous ECM molecules and some serum proteins contribute to the formation of peritubular dentin

2. INTRODUCTION

A thick dentin layer forms the bulk of mineralized dental tissues. Dentin is capped by a crown made of highly mineralized and protective enamel, and in the root, it is covered by cementum, a structure implicated in the attachment of the teeth to the bony socket. Teeth contain dental pulps in their central parts, and they are usually non-mineralized. This soft connective tissue also contains nerves and a vascular network connected with the surrounding tissues, the periodontal ligament and the bony socket. Taken as a whole, the general composition of dentin is summarized in Table 1.

On a weight basis, dentin is less mineralized than enamel (96% in weight), but more than bone or cementum (about 65% in weight).

However, this global distribution provides an oversimplified view, because dentin is a puzzle of different types of dentin, reflecting different functions and bearing their own specificities.

3. DENTIN: STRUCTURE AND ULTRASTRUCTURE – THE THREE-COMPARTMENTS MODEL

Physiologically and anatomically, dentin is a complex structure. Within what is named a “whole dentin”, different types of dentin have been identified, even within a single species.

Phylogenetic studies have revealed that, during evolution, dentin analogues were originally very similar to bone, with osteoblast/odontoblast-like cells located within alveoli, which is the case for osteocytes surrounded by bone within lacunae (1,2). This organization, called osteodentin, is still observed during tooth development in some mammalian species such as rodents (3), and as reparative dentin in humans. Odonotoblasts polarize, elongate and start to display two distinct parts: a cell body and a process. During the next step of evolution, the cell bodies are located outside the mineralized tissue, along the border of the mineralization front, and long processes occupy the lumen of dentin tubules. In contrast with bone, dentin is not vascularized, except in some fish teeth where the existence of vasodentin or vascular osteodentin is well-documented (4). When mammalian odontoblasts become terminally polarized, they produce an orthodentin, with cell bodies located outside the predentin/dentin layer at the periphery of the pulp, and cell processes crossing the predentin and extending inside dentin tubules up to the dentin-enamel junction. Tubules are characteristic of orthodentin (Figure 1) The diameter of tubules varies between 2 and 4 micrometers. The number of dentine tubules is about 18000 and 21000 tubules per mm² (5). They are more numerous in the inner third layer than the outer third layer of the dentin.

3.1. Peripheral outer layers

With some variation, most mammalian species have an outer mantle dentin layer, 15-30mm thick, at the periphery of the tooth in the coronal region. This is mainly an atubular layer, made up of few thin and curved tubules. In the root, similar layers are identified as follows: 1) a Tomes granular layer, formed by calciospheritic structures that have not fully merged, and consequently by interglobular spaces, and/or 2) the hyaline Hopewell-Smith layer, each 15 – 30 micrometers in thickness. The network of porosities, probably formed by organic remnants, is vertically oriented, and unrelated to the tubules (6). The functions of these peripheral layers have not been clearly established. However, some hypotheses may be drawn from their physical and chemical properties.

In the crown, the so-called “mantle dentin”, indentation measurements using Vickers microhardness show a gradual increase in hardness along the outer 200mm (7). This outer layer is less mineralized, and consequently the resilient mantle dentin may be adapted for dissipating pressures or forces which otherwise would induce enamel fissures and detachment of the fragmented enamel from the outer dentino-enamel junction. Dentin tubules are missing or reduced in number and bent in these layers of outer dentin. For some years, there was a debate about the observation that when using the “stains all” staining method (8) or antibodies raised against dentin phosphoproteins (9), the mantle dentin was unstained in contrast with the circumpulpal dentin, and consequently Takagi and Sasaki suggested that this layer was deprived of phosphorylated proteins (8) However, chemical analysis revealed that proteins usually phosphorylated are actually present but either in an underphosphorylated form or not phosphorylated at all (10). In this context it is interesting to note that the mantle dentin is unaffected in X-linked hypophosphatemia (11) whereas, in contrast, rickets are produced within the circumpulpal dentin enlarged non-mineralized interglobular spaces containing dentin ECM molecules (12). Hence, the nature of the non-collagenous proteins (NCPs) differs between the mantle and circumpulpal dentins, with the outer layer remaining uninfluenced by the organic phosphorous status. As a consequence, the outer layers in the crown are less mineralized than the rest of the dentin. They display some elastic properties and therefore provide some resilience, which is important from a mechanical point of view and allows dissipation of stress forces (7). However, in human, the thickness of the outer layer is about 200mm, which is larger than the presumed width of the mantle dentin.
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In the root of the tooth, the elastic modulus is again not uniform and the outer part provides a frame that may resist axial and lateral pressures (13). From a structural point of view, dentin tubules are scarce in the outer dentin, and lacking in many cases. Bent and narrow tubules are deviated by calciospheritic structures present in the granular Tomes’s layer.

At this early stage, it is worth noting that polarizing odontoblasts parallel with the basement membrane display some buddings. These membrane protrusions detach and become matrix vesicles very similar to those described in cartilage and bone, and these vesicles are thought to contribute to the initial dentin mineralization (14). This is the only instance in which a cell-derived organelle participates directly in mineralization during dentinogenesis. For the most part, the cells produce the extracellular matrix that is involved in the mineralization process.

3.2. Circumpulpal dentins

The circumpulpal dentin forms the largest part of the dentin layer. Thin during the initial stages of dentinogenesis, its thickness continuously increases (about 4 micrometers/day) at the expense of the space initially occupied by the pulp. Again, it is not a homogeneous dentin layer. The most prominent part of the circumpulpal dentin is formed by intertubular dentin, whereas peritubular dentin is found around the lumen of the tubules (Figures 1 and 2). The ratio between inter-tubular and peri-tubular dentin is highly species-dependent. Peritubular dentin is missing in the continuously growing rodent incisors. In contrast, in horses, the ratio is roughly 50%, and decreases in humans (about 10-20%), with huge variations depending on the area where the calculations are made.

Differences in the structure and composition of the two dentins are well documented. Type I collagen is the major protein of intertubular dentin (90%), whereas no collagen fibrils are observed in the peritubular dentin. Differences have been also reported in the composition of non-collagenous proteins of the two dentins (15-18). Along these lines, some crystallo-chemical specificities of the inter- and peri-tubular dentins have been established.

In intertubular dentin two parallel plates form the plate-like crystallites, 2-5 nm in thickness and 60nm in length. At lower magnification, inter-tubular crystallites have a needle-like appearance (Figure 2). They are often located at the surface of the collagen fibrils, parallel to the collagen fibril axis; otherwise, the crystallites randomly fill interfibrillar spaces (19). Demineralization of intertubular dentin reveals a dense network of collagen fibrils, coated by non-collagenous proteins (Figure 3). Glycosaminoglycans, stained by cationic dyes, are associated with the collagen hole zones resulting from the overlapping of collagen subunits.

In the peri-tubular dentin, isodiametric structures about 25nm in diameter have been reported (15, 20). At a higher resolution, the crystals are bearing the following measurements: a= 36nm, b=25nm and c= 9.75nm (21).
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Figure 4. Staining ultra-thin sections with phosphotungstic acid/chromic acid reveals a dense network in the peritubular dentin (pt) Intertubular dentin (it) is differently organized as a result from the subjacent collagen network. Lu = lumen of the tubule.

Figure 5. A: Peritubular (pt) electron dense alcian blue stainable material located around the lumen (Lu) of the tubule. In the peritubular dentin (pt), after mild demineralization, an alcian blue-positive material is observed as a network. The mesh of the network is about 30 nm wide. Lu = lumen; it = Intertubular dentin. B: The lumens (Lu) of the tubules are partially filled by crystals contributing to the formation of sclerotic dentin (sd). Pericanalicular dentin (pc)

They form a ring around the lumen of the tubules. After mild demineralization, no collagen fibrils are detectable, but a thin network of non-collagenous proteins and phospholipids are visible (15, 18, 19) (Figures 4 and 5).

During odontogenesis, odontoblasts are critical for the formation of a primary dentin until the tooth becomes functional. When contacts between antagonistic cusps are established, then the formation of secondary dentin starts immediately and continues throughout life. Initially, odontoblasts constantly produce matrix molecules that result in the formation of a layer 10 micrometers thick, reduced afterward to a daily deposit of 4 micrometers. However, there is not much difference between primary and secondary dentin. The only major difference is morphological, and the S-curve of the tubules is more accentuated in the secondary dentin, due to the gradual space restriction of odontoblasts located at the periphery of a withdrawing pulp.

The concomitant formation of inter- and peritubular dentin results from two different types of mineralization. Inter-tubular dentin results from the changes occurring between a dynamic non-mineralized predentin and the dentin located behind the mineralization front, a border that we now call metadentin (22). Polarized odontoblasts are formed by a cell body that is key for synthesis of the components of extracellular matrix molecules (ECM), along with a long process wherein the secretion of ECM takes place both in predentin and dentin. Processes are also implicated in the re-internalization of some fragments after the degradation of some ECM molecules. Some major ECM molecules, namely collagen and proteoglycans (PGs), are secreted in the predentin, whereas other ECM molecules are secreted more distally near the mineralization front, or even further within the lumen of tubules. In the proximal predentin, odontoblasts are responsible for the secretion of native type I collagen, together with some PGs (decorin (DCN), biglycan (BGN), lumican (Lu), fibromodulin (Fmod)) implicated in collagen fibrillation (23, 24). Some NCPs are implicated in the nucleation and growth of the mineral phase, or in its inhibition. Most phosphorylated proteins are secreted in the metadentin, near the mineralization front. In the proximal predentin (near the cell bodies) the mean diameter of collagen fibrils is about 20 nm, whereas in the central part the mean diameter reaches 40 nm, and in the distal part, near the mineralization front, fibril diameter varies between 55-75 nm (25). This suggests that the increased diameter is due to lateral aggregation of collagen subunits (26). In contrast, in the mineralized dentin, the diameter of the collagen fibrils is stable.

The formation of inter-tubular dentin provides a unique three-layer model, very convenient to study matrix-derived mineralization. Anatomically, there are three successive layers: 1) the cellular stratum (odontoblast cell bodies and Höehl’s cells (27), located at the periphery of the pulp), 2) the immature predentin layer, with a constant 15-20 micrometers thickness, and 3) the mineralized dentin, starting at the mineralization front up to the mantle dentino-enamel junction. This model is similar to the compartmentalized bone model, where three parts are also found: the osteoblast/bone lining cells layer, osteoid and bone. This observation may shed light on processes shared by bone and dentin. However, while there may be some similarities between bone and tooth formation, it is also clear that they have unique properties. For example, bone formation is followed by a constant remodeling due to osteoclast-osteoblast interactions, hormonal influences and matrix metalloproteinase (MMP) degradation of the existing matrix proteins, whereas after its formation, dentin is quite a stable structure.

Peritubular dentin does not result from such transformation of predentin into dentin, but rather from the adsorption along the lumen of the tubules of an amorphous
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Matrix. It may be secreted by the odontoblast processes within dentin, or take origin from the serum (dentinal lymph) Proteoglycans, lipids and other ECM proteins are implicated in the formation of a thin amorphous network, giving rise to a dense hypermineralized peritubular dentin. In two species, the elephant and the opossum (Didelphis albiventris), the formation of peritubular dentin occurs prior to intertubular dentin, with prominent calcospheritic structures present at the mineralization front (28, 29). In the other species studied so far, the formation of peritubular dentin occurs within the tubules some distance away from the mineralization front, and is mostly developed in the two inner parts of the circumpulpal dentin.

After eruption, as a reaction to carious decay or to abrasion, reactionary or tertiary dentin is formed beneath a calcitroumatic line, which is interpreted as an interruption of normal dentinogenesis (Figures 5 and 6). This dentin is relatively unstained by the "stains all" method, and is therefore deficient in acidic proteins, some of which are presumed to be phosphorylated. As is the case for the mantle dentin, this finding is due either to a defective post-translational modification (i.e. phosphorylation), or to the absence of the acidic proteins. Reactionary dentin appears either as a layer of the osteodentin type, or as a tubular or atubular orthodentin, depending on the speed and severity of the carious attack, the progression of the reaction and the age of the patient. Such dentin may also be a physio-pathological response to abrasion, reactionary or tertiary dentin is formed beneath a calciotraumatic line, which is interpreted as an  

To conclude this section, we are going to discuss why, depending on the type and the location of the dentin, at least three different physiological mineralization processes occur. Firstly, the dentin outer layers result from cell-derived events involving the presence of matrix vesicles and their enzymatic equipment. This process may or may not be associated with odontoblast apoptosis. Secondly, the active transformation of predentin into dentin is the origin of intertubular dentin formation. This is a matrix-controlled process, and involves type I collagen playing a major role, at least as a non-collagen proteins carrier. Thirdly, a passive deposit of serum-derived molecules along the tubule walls leads to the formation of peritubular dentin. This is why, under a generic name of dentinogenesis, different types of mineralization produce very different tissues. The unanswered question is why it is necessary to have three totally different processes involved in the formation of dentin in a single tooth. Subsequently, this leads to the question, what are the determinants for selecting one of the three pathways or why one over other?

4. ODONTOBLASTS: IMPLICATION IN THE SYNTHESIS, SECRETION AND MINERALIZATION OF DENTIN ECM

4.1. Odontoblasts and dentin formation
4.1.1. From pre-odontoblasts to pre-secretory polarizing odontoblasts:

During the early stages of odontogenesis, cells originating from the neural crest migrate toward the para-axial mesenchyme, and reach the territory of the first branchial arch where they contribute to the formation of tooth buds. In the maxilla of the median region, odontoblasts’ precursors migrate from the fronto-nasal bud. Interactions between epithelial cells of the dental lamina and mesenchymal condensations contribute to embryonic pulp formation. During the period of migration, the pre-odontoblasts proliferate and a fixed number of divisions allow these cells to reach the periphery of the dental pulp. During the last mitosis, the daughter cells located near or in contact with the basement membrane (BM) become presecretory prepolarized odontoblasts. The daughter cells located some distance away from the BM form the Höehl’s layer. Initially, these cells seem to be non functional, but they may later constitute a reservoir for the renewal of old odontoblasts destroyed by apoptotic processes (30).

4.1.2. Odontoblasts differentiate and become functional:

When odontoblasts are differentiated, they undergo terminal polarization. Four events then occur: 1) The migration of the Golgi apparatus from the basal part to a supra-nuclear area, 2) The development of cytoskeletal proteins, microtubules and cillum, actin microfilaments, and vimentin and nestin-containing intermediate filaments, 3) The formation of a junctional distal complex comprising desmosome-like junctions, gap junctions and, in some

Figure 6 A: Beneath a calciotraumatic line reactionary dentin is formed as a response to a carious lesion. Odontoblasts (o),predentin (pd) and pulp (p) bear normal appearance. B and C: Reparative dentin (white star) forms in response to the implantation of bioactive molecule within the exposed pulp (p) after preparation of a cavity (c and arrow)
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Figure 7. A. initial stage of mantle dentin formation. Odontoblasts (o) and ameloblasts (a) are involved in the formation of a basement membrane (arrowheads) B. Beginning of polarization of secretory odontoblasts (o). The distal junctional complex is not yet formed.

Figure 8. A. in the cell body of the secretory odontoblast, the pathway for synthesis involves the rough endoplasmic reticulum (RE), the Golgi apparatus (GO) Mitochondria (M) produce the energy for intracellular transfer. The secretion is under the control of post-Golgi vesicles (VPGO) and complex multivesicular structures (CMV). B. Cytoskeletal proteins such as microtubules (MT) play role in the terminal polarization of the cells and cilium organization.

Figure 9. A and B. Odontoblast processes (o) crossing the predentin (pd) and reaching dentin (d).

species, tight junctions. These junctional complexes constitute a solid permeability membrane, and intercellular diffusions are restricted to molecules with small molecular weight, and 4) Fenestrated capillaries infiltrate the odontoblast layer. Amino acids, fatty acids, sugars and ions, as precursors of intracellular and ECM molecules, cross the space between endothelial cells and the BM. They are incorporated within odontoblasts (25, 31) (Figure 7).

Odontoblasts in their terminal cell division are at first roughly parallel to the BM, but after a short period their great axis is at right angles with the BM. Odontoblasts are aligned at the surface of the dental pulp. They form a palisade-like structure. The terminal polarization leads to the partition between i) A cell body where all the organelles implicated in ECM synthesis are present, including rough endoplasmic reticulum, the Golgi apparatus, and immature and mature secretory vesicles, associated with lysosomal equipment (GERL, small and large lysosomal vesicles, multivesicular structures) (Figure 8), and ii) A long process protruding in the predentin (Figure 9) and adhering to the dentinal walls of the tubules (Figures 9 and 10). The question of the length of the process remains unanswered. Formerly, it was assumed that the processes reach the dentino-enamel junctions (DEJ). The first data obtained with the Transmission Electron Microscope after heart perfusion of the fixative solution established that in the cat, processes are located in the inner third, and never extend more than half the distance between the pulp and the DEJ. Immunocytochemical data reveal that cytoskeletal proteins are found up to the DEJ, and thus the process may extend up to that interface. It is possible, however, that the processes withdraw but some non-functional remnants of the process remain, adhering to the tubule wall (31).

4.1.3. The functional odontoblasts: synthesis and secretion of extracellular matrix components

4.1.3.1. Collagen

Radioautographic data using labeled amino acids such as 3H proline have clarified the mechanisms of incorporation and the fate of this major collagen precursor and hence the synthesis of collagen (25, 32, 33). As early as 5-30 min after the injection, silver grains are visible mostly within odontoblast cell bodies, in a central area containing rough endoplasmic reticulum and Golgi cisternae. A few silver grains are present in the cell processes in the proximal predentin. Between 1-2 hours, the number of silver grains increases in predentin, namely in the proximal and central parts. Between 4-6 hours, the different parts of the predentin are totally filled with silver grains, whereas the percentage of silver grains scored over the cell bodies decreases (25) [See Table 2] (Figures 11-13).

After 24 hours, the labeling is very weak in odontoblasts, is firmly reduced in predentin and then only an accumulation of silver grains is seen forming a dense band 10-20 micrometers in width at the mineralization front in the inner dentin side (34). Later, the band is covered by the dentin newly formed during the next 48h and more, which is not labeled. This labeling is stable and remains even after longer periods of time. There is no translocation of the labeled band, at constant distance from the dentino-enamel junction.
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Table 2. Percentage of silver grains in odontoblasts, predentin and dentin (25)

<table>
<thead>
<tr>
<th></th>
<th>30 min</th>
<th>2h</th>
<th>4h</th>
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<tr>
<td>odontoblasts</td>
<td>70.5%</td>
<td>36.5%</td>
<td>27.0%</td>
</tr>
<tr>
<td>predentin</td>
<td>29.5%</td>
<td>63.5%</td>
<td>73.0%</td>
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Figure 10. Odontoblast processes in the predentin (pd) A and D. TEM, B and C. Freeze fracture replica. In C, arrowheads indicate membrane sites where endocytosis is initiated. In D, coated vesicles (cv) seen on ultrathin sections are implicated in the re-internalization of spliced molecules.

This cascade of events corresponds firstly to the synthesis of procollagen fibrils in odontoblasts' cell bodies. The cells incorporate proline (~119 amino acids /thousand residues in collagen analysis) and hydroxyproline (96 aa /thousand residues) in the pro-alpha 1 and pro-alpha 2 chains (35). Secondly, end-to-end elongation and lateral aggregation (26) contribute to the increased diameter of the fibrils, and their migration throughout the predentin toward the place where they undergo mineralization (Figure 11). The time response provided by these reports established that the newly synthesized collagen migrates in predentin, and therefore it is a dynamic process. At some stage of maturation (probably when cross-links provide stability to the collagen fibrils), they are incorporated and immobilized in the forming circumpulpal dentin (Figures 12-13).

Radioautographic data and experiments using microtubule inhibitors suggest the occurrence of a flux of forces in predentin and the active transport of collagen fibrils from the proximal to the distal predentin where the mineralization process occurs (25).

4.1.3.2. Phosphorylated proteins

Using [3H] serine and [33P] phosphate as phosphoprotein precursors, Weinstock & Leblond (36) showed that, in the rat, after 30 min silver grains are seen over odontoblasts cell bodies, in the Golgi region. The major difference with [3H proline] incorporation is that after 90 min, silver grains are located in the proximal predentin and a labeled band appears over the edge of dentin. After 4h, predentin is labeled in the middle region and in the mineralization front. This clearly shows that the band seen at the mineralization front is not resulting from the transformation of a labeled predentin into dentin, but that there are concomitantly two secretory places where labeled molecules are released. As the same silver grain distribution is seen with [33P] phosphate, it was concluded that the pattern of incorporation within a phosphoprotein differs from what has been established for collagen. Indeed 48% of the serine residues are incorporated into collagen, whereas 52% are included in phosphoprotein. This provides a clear-cut explanation for the two distinct pathways evidenced by radioautographic data. After incorporation within the pro-collagen chains, it takes 6h for collagen fibrils’ translocation to move toward the mineralization front. Intra- or extra-cellular diffusion of the labeled acidic phosphoprotein is more rapid and the protein is apparently secreted distally in the predentin, in the place where mineralization occurs. Electron histochemical observation of ultra-thin Epon sections stained with a phosphotungstic acid/chromic acid mixture, or using the “stains all” method with the light microscope, shows both stainings at the mineralization front. In both cases the staining is missing after pretreatment of the sections with alkaline phosphatase (37, 38). This substantiates the occurrence of dual labeling in the three compartment model, the secretion of newly synthesized collagen occurring in the proximal predentin, whereas the mineral-associated phosphorylated protein is released near the predentin/dentin junction (Figure 11-13).

This dual secretion is supported by other experiments related to proteoglycans’ (PGs) incorporation within predentin and dentin. Histochemical methods using cationic dyes or cationic detergents show that glycosaminoglycans (GAGs) are associated with collagen fibrils in predentin (37, 39, 40). Immunostaining with chondroitin sulfate/dermatan sulfate (CS/DS) antibody (2B6) also reveals intensive staining in predentin (40, 41). In addition, the occurrence of gradients of distribution...
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Figure 12. A: After rapid-freezing high pressure physical fixation the junction (metadentin, md) between predentin (pd) and dentin (d) looks porous, with incomplete mineralization between mineralizing collagen fibrils. B: Unstained ultra-thin section junction between predentin (PD) and dentin (D). C: The junction between predentin and dentin is underlined after PTA/chromic acid staining.

Figure 13. Upper part of the figure: Demineralized intertubular dentin. Thick collagen fibrils with a characteristic periodic banding form a dense network. Intercollagen spaces (ics) play a role in the construction of the tissue. Lower part of the figure: Light microscopy radioautography after 3Hproline injection. At 30 min silver grains are located at the junction between the odontoblast cell bodies (O) and the predentin (A), After 2h, silver grains are located in the proximal part of the predentin (PD) (B) Four hours after the injection of the labeled proline, (C) silver grains are located throughout the whole predentin thickness. Cp: capillary

between CS/DS and keratan sulfate (KS) (42) supports functional differences between the two groups of GAGs. It was quite clear that an expanded amorphous gel, rich in PGs as revealed by its GAGs content, forms the ground substance between the collagen fibrils (43). After the fixation procedure combined with cationic dyes, and after the subsequent coiling during the shrinkage due to dehydration, the GAGs appear as stellar or boomerang-like structures located along the collagen fibrils, or in close association with the so-called holes due to the quarter stagger structure of the fibrils (39) (Figure 13).

4.1.3.3. Glycosaminoglycans and proteoglycans

In this context, after [35S] sulfate injection, a time course study revealed a dual localization of the labeling (44). One part rapidly incorporates into the predentin, eventually moving between 2 to 4h from the proximal to the distal part of this compartment. The labeling is gradually reduced and nearly disappears after 120 h. Another zone of secretion is revealed by a concomitant labeling, displaying a totally different location. As early as 30 min, the mineralization front begins to be labeled. The labeling is increased at the dentin edge between 1 and 2 hours and reaches a maximum at 4 h. The labeled band is slightly reduced afterward, but remains high, being gradually incorporated into the dentin, and gradually covered by newly formed layers of unlabeled dentin. The density and the distance to the dentino-enamel junction remain stable, even at 120h. This supports the existence of two distinct groups of PGs. One group located in predentin is probably related to collagen migration and fibrillation. These predentin PGs may constitute a substrate for stromelysin (MMP-3) They are degraded and disappear from this compartment (42). They were further identified as CS/DS proteoglycans (45) and KS proteoglycans (46). Indeed the diameter of collagen fibrils is regulated by these proteoglycans, as shown by measurements carried out using biglycan- and fibromodulin-deficient mice (23, 24). The second group of small PGs is secreted near the predentin-dentin junction and is apparently associated with the mineral phase. The incorporation of [35S] sulfate into dentin PGs is stable in time and place.

> These radioautographic investigations provide evidence that odontoblasts are important in the production of the predominant ECM molecules synthesis. They also shed light on the occurrence of two distinct zones of secretion. [3H] proline is incorporated into secretory granules between 30 min and 1h, but no labeling is found at the mineralization front before 45 min. Therefore, the band appearing gradually after 6h in dentin results from the eventual incorporation of labeled collagen fibrils into the dentin compartment. In contrast, [3H] serine and [35S] sulfate display significant labeling at the mineralization front as early as 30-45min. This implies that the labeled molecules are secreted at the junction between the distal predentin and the mineralization front. It is still unclear if odontoblasts’ processes are involved in the second flux of secretion. Once released in the proximal predentin, diffusion of the precursor may occur independently and rapidly throughout predentin without direct implication of odontoblasts’ processes (44, 47). This question is not clarified by [3H] fucose labeling, at least for the fucose-containing glycoproteins, slightly diverging from the results having been previously published. Four hours are necessary before accumulation of silver grains is seen at the mineralization front (48), whereas in another publication, 1h after the injection labeling starts to develop at the predentin-dentin junction, which is densely labeled at 4h (49).
4.2. Inter cellular pathway: Phospholipids and albumin as lipid carrier, calcium transfer.

While exploring the role of odontoblasts in dentin formation and mineralization, we sensed that there are cases in which the incorporation of some ECM components may follow intercellular pathways independently from any cell control. When [\(^{3}H\)]choline was injected intravenously, radioautographic data shows that as soon as 30 min after the injection some labeling was seen in dentin, whereas the labeling was not above background in odontoblasts and predentin. The labeling seen throughout dentin was reinforced at 1h. It took ~ 2h to see a significant labeling above the background in the cells, and in the predentin as well. This provided some proof that odontoblasts may not be solely responsible for the transfer and incorporation of phospholipid precursors into dentin. Grain density peaked at 24h and diminished at 4 days (50). Isotopic exchanges in the blood between labeled cell membranes and the serum may take place within a few seconds and contribute to the intercellular diffusion. The possible occurrence of such cell-independent diffusion adds a third possibility to the two previously reported cell-controlled pathways. This implies that most but not all ECM molecules are regulated by odontoblasts’ activities. As revealed by fluorescent tracers, a transjunctional flux is facilitated by the presence of gap and desmosome-like junctions in intercellular contacts between odontoblasts (51).

Another experiment also supports the concept of intercellular diffusion. Rabbits injected with [\(^{125}\)I]albumin were euthanized at 1h, 6h and 3 days after injection. After processing for radioautography, silver grains were seen in predentin after 1 h and in dentin after 6h. Interestingly, no labeling was detected in enamel. The structures present in the junctional complexes between odontoblasts (gap and desmosome-like junctions) and ameloblasts (tight and gap junctions) may account for the difference in the diffusion process (52). Albumin is considered a lipid-binding protein (53). It is not synthesized by odontoblasts, but may be found in dentin.

Interestingly, electron microscopic autoradiography of [\(^{45}\)Ca] (54, 55) substantiated the likelihood of two transfer pathways. The first and major one occurs through the intercellular spaces between odontoblasts, with [\(^{45}\)Ca] directly reaching the mineralization front at the dentin edge within 30 sec to 5 min after injection and lacking any detectable radioactivity in odontoblasts (54). A second transcellular transfer was evidenced through the odontoblasts, demonstrated by cytoplasm labeling of the odontoblasts’ processes and a dense labeling of the mineralization front, but only 6h after the injection (55).

To conclude, the role of odontoblasts in the synthesis and secretion of ECM components is crucial in dentinogenesis. Depending on the labeled precursor, secretion either occurs in the proximal predentin or at the distal predentin - inner dentin edge. The two different sites are related to either the release of collagen fibrils and their associated proteoglycans in predentin, or to the discharge of non-collagenous phosphorylated proteins and mineral associated proteoglycans that are secreted at the mineralization front or metadentin, respectively (22). Some matrix components migrate directly from the serum to the dentin compartment. They mainly follow an intercellular pathway, albumin and phospholipids being implicated in the transport of minerals toward intertubular dentin, and therefore in its mineralization process.

5. GLOBAL COMPOSITION OF THE EXTRACELLULAR MATRIX

Table 3 lists the extracellular matrix components extracted from the different types of dentins. As structural proteins, ECM molecules are implicated in the formation and mineralization of dentin. They may act either as promoters or as inhibitors, depending on concentration and post-translational modification. Some of them are characterized as matricellular molecules. This means that they serve as biological mediators of cell functions or modulators. They interact with cells, namely cell surface receptors and integrins, or they modulate the activity of growth factors and proteases. They determine specific downstream effects on gene expression and cell phenotype (57). In dentin, SPARC, osteopontin (OPN) and bone sialoprotein (BSP) are identified as matricellular proteins, but other molecules seem to play a similar role. ECM molecules may also be implicated into cell signaling (58). These three properties interact and make classification difficult.


Calcification results from physico-chemical interactions between calcium and phosphate leading to the formation of a mineral phase in soft tissues. It can have both positive and negative outcomes, including ectopic calcification, vascular calcification and kidney stone formation. Calcification may occur as a result of hypercalcemia, or may be induced by post-necrotic events. Spontaneous precipitation of mineral complexes may also occur, but generally not in an apatitic form. Often, terminology is not always used accurately. Calcification is usually a non-physiologic event; mineralization is generally physiologic. Biomineralization, in general, is the process through which cells orchestrate the deposition of minerals. In vertebrates, biomineralization is the cell-mediated process by which hydroxyapatite (HA) is deposited in the extracellular matrix (ECM) of skeletal structures. Structural molecules of the ECM and a series of enzymes direct the entry and fixation of mineral salts exclusively in bone and mineralized dental tissues.

During dentinogenesis, at least three different sites of mineralization are identified: 1) mineralization driven by the cell derived-matrix vesicles and occurring mainly in the mantle dentin, 2) ECM molecule-derived mineralization, accounting for the majority of dentin formation, and 3) blood-serum-derived precipitation occurring in the peritubular dentin. Although substantial differences are recognized between the three types of
mineralization, there are some common features that allow for the proposal of a general concept regarding the mechanisms involved. The matrix-nested biomineralization of intertubular dentin has been extensively studied and provides a good model.

Mild demineralization, specific staining and other methods have shown the presence of organic sheaths or envelopes present exclusively at the surface of the crystallite, at least in dentin. They have been reported in the literature as “crystal ghosts”. These structures combine proteins, proteoglycans and phospholipids, and they have been identified as phosphoglycolipid proteins. As an enzymes-rich or enzymatically inactive envelope, they are structurally related to the so-called extracellular matrix vesicles that are present at the onset of mantle dentin formation in teeth and at later stages, during the onset of pulp repair. They are also present in some embryonic bone and calcifying cartilage, identified as loci where mineralization is initiated (59, 60). Although originally suggested to be derived from apoptotic bodies, recent studies indicate that matrix vesicles are not apoptotic in origin (62). Differences in the activities of enzymes associated with matrix vesicles and crystal ghosts are well recognized, but the general mechanisms through which they affect mineralization may be similar.

As a working hypothesis clarifying the cascade of events leading to mineralization in the collagen-based tissues, a series of steps have been indicated. The initial cell-mediated events are matrix-dependent. Fibronectin, and then collagen, are deposited. Non-collagenous proteins are post-translationally modified by casein kinases (phosphorylation), protein phosphatases (dephosphorylation), bone matrix protein 1 (BMP1) and PHEX (fragmentation), and sulfatases (sulfation). Ca++ ions interact with the acidic residues and furthermore combine with PO4 to initiate the nucleation process. The collagen matrix provides a spatial template upon which the mineral crystals deposit. The growth of these crystals is also directed by the ECM proteins. (64).

5.2. ECM molecules implicated in dentinogenesis:

There have been a variety of attempts made to identify the proteins and other matrix components implicated in dentinogenesis. These range from protein (31) to gene expression studies (65), to gene discovery studies defining the dentinogenesis transcriptome (66)

5.2.1. Genes coding for dentin ECM

Among the genes expressed in rat incisors, the most abundant code for osteonectin, alpha1 (I) and alpha2 (I) collagen, and DCN (65). Osteoadherin, PHEX, dentin sialophosphoprotein (DSPP), bone sialoprotein (BSP) and enamelysien are also expressed in human odontoblasts (67).

However there are also other genes that are expressed by odontoblasts and seem to be important in the context of dentinogenesis. These genes code for the so-called Small Integrin-Binding Ligands, N-linked...
Glycoproteins (SIBLING) proteins, the non-phosphorylated non-collagenous proteins (e.g. osteocalcin (OC)) and for some members of the Small Leucine-rich Proteoglycans (SLRPs) family. Many other ECM molecules listed in table III are present within the cells or in the matrix. They may also contribute to some aspects of dentinogenesis, but either their expression is very weak, or they are more ubiquitous and may not be primarily involved in this process. In addition, some molecules originate in the blood serum and are not synthesized by odontoblasts.

There is a high probability that during the last 50 years, almost all the major dentin ECM molecules have been identified. During this time, attempts were made to characterize the genes coding for the major molecules, to establish the three-dimensional structure of the protein and to discover their functions. The goal originally was to identify the target molecule(s) that play a key role in dentin biomineralization. Because similar molecules are present in bone and dentin, they may have the same function. However, in contrast to bone, there is little or no remodeling in dentin. Consequently, dentinogenesis provides an excellent model to study the biomineralization processes of skeletal tissues.

This goal was broadened and it is now clear that ECM molecules are multifunctional, being structural, matricellular and signaling molecules. They are constantly interacting and being up- or down-regulated by other ECM molecules. So, the identification of a single “master regulator” molecule is naive and clearly more complicated. It is obvious that the effects are modulated by the concentration of the molecule. In addition to the dose-dependency, the time period allowing interaction between the effectors and potential receptors is an important factor modulating the biological outcome. Emphasis has more recently been placed on the specific properties of certain domains of the molecule, which are exposed or hidden in some presentations of the molecule. Many of the ECM proteins are intrinsically disordered (68), having flexible structures that vary as they interact with their miscellaneous partners (other proteins, enzymes and minerals). These Intrinsically Disordered Proteins (IDPs) include osteopontin and bone sialoprotein (69, 70), dentin matrix protein 1 (DMP1) (71) and amelogenin (72). In light of the concept of cryptic sites and IDPs the role of matricryptic sites has been reported (73). Some signals are derived from biologically active cryptic sites, revealed after conformational alterations of the molecule. ECM denaturation provides new signals to regulate the tissue response, including the mineralization process.

5.2.2. Type I Collagen

Collagen is the major protein found in dentin. It constitutes ~90% of the organic matrix. The majority of the collagen is type I, although trace amounts of type III and V have been reported (35). Type I collagen results from the self-assembly of two alpha 1 (I) chains and one alpha 2 (I) chain (89%), whereas 11% is formed by three alpha 1 (I) chains. These chains are assembled in a triple helix with a coiled coil conformation (74).

About 3% of the collagen fibrils are composed of type III and/or type V collagens. This is found in cell cultures, in very young animals, or when defective collagen synthesis occurs.

Fibrillar growth is primarily due to the lateral self-assembly of fibril subunits, followed by a linear fusion implicated in collagen lengthening (75). Collagen fibrillogenesis is influenced by dentin small leucine-rich proteoglycans (76). Removal of GAGs delays fibrillogenesis. PGs inhibit fibrillogenesis and fibrils’ thickness over concentrations of 50-25 micrograms/mL.

Collagen is synthesized and subsequently controlled by the odontoblasts. The assembly of pro-collagen chains is initiated within the rough endoplasmic reticulum, glycosylation and sulphatation occur in the Golgi apparatus, and procollagen maturation occurs in early secretory vesicles. Tiny fibrils accumulate in these vesicles. Vesicles containing abacus-like structures have been interpreted as being the mode of intracellular transfer of pro-collagen. However, it has been noted that their number is increased when secretion is impaired by pharmacological drugs such as vinblastine or others vinca alkaloids, or colchicines, a family of drugs that act on the polymerization of cytoskeletal proteins, namely tubulin (77). It was shown that the abacus-like structures are acid phosphatase-containing structures, and therefore lysosomes (78, 79). This is the place where defective collagen fibrils are destroyed and not transported to the place where they are normally secreted.

Procollagen non-helical extensions are cleaved by procollagen peptidases, and the fibrils transformed into native collagen. Interestingly, the shorter 70kDa form of the C-proteinase is identical to Bone Morphogenetic Protein-1 (80).

In the rat incisor, the fibrils just secreted in the proximal predentin are parallel to the mineralization front, and undergo lateral aggregation. The diameter in the proximal third of predentin of the fibrils is about 20 nanometers, 40 nanometers in the central third, and 55-75 micrometers in the distal part, near the mineralization front. In dentin, the diameter varies between 100-120 nanometers. In the proximal predentin, 88 ± 4% of the new collagen fibrils are parallel to the mineralization front. In the central part, according to our calculations 74.5 ± 6% have the same orientation, but only 62±8% of those are in the distal third. This suggests that the fibrils are pushed toward the place where mineralization occurs. A flux of forces is involved in this transfer, which may be altered by the injection of vinblastine, which freezes synthesis and secretion. In such in vivo experiments, one fibril in four loses this orientation. Consequently, the orientation of the collagen fibrils becomes random. (25) (Figure 11).

The transfer of collagen fibrils within the predentin from the proximal to the distal parts assumes that an amorphous ground substance serves as a viscous gel allowing the fibril’s translocation. Observation after rapid freezing and freeze substitution shows that, in predentin, the glycosaminoglycan-rich interfibrillar matrix is amorphous and therefore may fulfill this requirement (43) (Figure 16).
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Figure 14. A. Stains all staining revealing phosphoproteins is mostly positive near the dentin-predentin junction. B. Von Kossa staining revealing the phosphate and calcium ions is dense over dentin C. Radiolabeling using ³H serine as labeled precursor underlines the mineralization front (mf) Here at 4h the labeling is strong at the junction between predentin (pd) and dentin (d). D. Phosphotungstic acid /chromic acid staining reveals marked electron density in metadentin at the junction between predentin and dentin, at the dentin edge. Right part of the figure: Immunolabeling with an anti-dspp stains the predentin/dentin junction and odontoblasts (o) at an early stage of formation of tooth germ. Dentin (d) and pulp (p) are weakly stained.

Figure 15. Anti MEPE labeling. Pulp cells (p), odontoblasts (o), predentin (pd), ameloblasts (a), enamel organ (eo) and bone trabeculae (b) are positively stained, but not enamel (e) and dentin (d).

The mean diameters of the collagen fibrils in mice molars are enlarged in the BGN- and Fmod-deficient mice, whereas no difference is detected between the wild type and DCN-deficient mice (23, 24). This confirms that certain PGs may regulate collagen fibrillation.

In the coronal part of the rodent molar, the organization of collagen fibrils is slightly different. In large areas, fibrils are broadly parallel, as is the case in the incisor, but each of these areas is limited from part and other by fan-like structures, inserted at right angles into dentin. This dual organization likely has a morphogenetic role and may contribute to a general architecture involving the formation of cusps. In the root, inter-odontoblastic collagen fibrils--the so-called von Korff fibrils--are also oriented at right angles to the dentin inner surface. They are aligned in strands, which may contribute to the gradual reduction in diameter of the root (81).

Altogether, it is clear that collagen fibrils are important in dentinogenesis, mostly because they provide an organized scaffold. Self-aggregating properties contribute to the formation of calcospherites or calcospherulites. However, in the absence of NCP, collagen fibrils do not appear to be directly involved in mineralization, and in this context, only an association of bovine dentin phosphoprotein with collagen fragments is effective (82).

5.2.3. Non-collagenous molecules

Phosphorylated proteins form a first group of molecules. The Small Integrin-Binding Ligand N-linked Glycoproteins (SIBLINGs) family seems to play a crucial role in dentinogenesis (56).

5.2.3.1. SIBLINGs

They have been initially found in mineralized tissues (bone and teeth), but they have also been identified in soft tissues such as salivary glands, kidney and prostate tumors.

In dentin, the SIBLING family includes DSPP (Dentin SialoPhosphoProtein), immediately cleaved after secretion into dentin sialoprotein (DSP), dentin glycoprotein (DGP) and dentin phosphoprotein (DPP). It also contains a dentin matrix protein-1 (DMP-1), a bone sialoprotein (BSP), osteopontin (OPN) and a matrix extracellular phosphoglycoprotein (MEPE). They are produced from the expression of genes located on chromosome 4 q21. Mutations of DSPP and DMP-1 genes lead to different forms of dentinogenesis imperfecta or dentin dysplasia, whereas mutations in MEPE are associated with X-link hypophosphaemia. The relationship between the protein mutations and dentin pathologies clearly reveals the importance of the SIBLINGs in dentinogenesis.

The distribution of SIBLINGs varies when analyzed by the 3-step mode of extraction (83):

1) The G1 extract is obtained by using a guanidium-HCl mixture. This allows the extraction of the NH2-terminal fragment of DSPP and its PG form, as well as BSP, OPN and the NH2-terminal fragment of DMP1.

2) The E extract is obtained after treatment of the tissue by EDTA solution, a procedure recognized for its ability to extract Non Collagenous Proteins (NCPs) associated with the mineral phase, identified as hydroxyapatite (HAp). In this extract, the NH2-terminal fragment and the COOH-terminal fragment of DMP-1 are found, in addition to BSP and OPN.

3) The G2 extract allows the detection of NCPs extracted by the EDTA solution procedure, followed by guanidium-HCl treatment. Only BSP is present in the G2 extract.
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Figure 16. Left part of the figure: ultrastructure of human X-link hypophosphatemic dentin. N. normal dentin, XLH hypophosphatemic dentin. Large empty interglobular spaces are detected between calcospherites. Enamel looks unaltered. The mantle dentin (md) is unaffected by the genetic disease, in contrast with the circumpulpal dentin (cd) displaying severe alterations. Right part of the figure After rapid freezing followed by freeze substitution, between electron lucent collagen fibrils, intercollagen spaces are filled by an amorphous electron-dense alcian blue stainable material, allowing the transport of collagen fibrils (co) between the places where they are secreted toward the mineralization front.

In addition, an association between some SIBLINGs and metalloproteinases (MMPs) has been established in soft tissues. BSP is associated with and functionally related to MMP-2; DMP-1 to MMP-9 and OPN to MMP-3 (84, 85). With regards to dentinogenesis, such partnerships may impact the cleavage of these structural molecules and subsequently expose new biologically active domains.

5.2.3.1.1. DSPP and its daughter molecules after cleavage

The Dentin SialoPhosphoProtein (DSPP) is expressed mostly in dentin (Figure 14), but not exclusively. The molecule has been detected in bone at only 1/400th of the level of dentin. The DSPP gene is located on chromosome 4 locus q22.1. It is cleaved immediately after secretion by MMP2 and MMP20 into three daughter molecules (86):

1) the Dentin Sialo Protein (DSP) – the N-terminal region of DSPP, with a Mw of 155kDa and a total length of 360-370 amino acids- aa 16-374

2) the Dentin Glycoprotein (DGP), with Mw around 19kDa (amino acids 375- 462) (87).

3) the Dentin Phosphoproteins (DPP) or phosphophoryn (155kDa in bovine teeth and 90- 95kDa in the rat, 72kDa in mice, 53kDa according to some authors in some species) It is the C-terminal region of DSPP (amino acids 463-1253) However, the cleavage into 2 natural dentin matrix products (DSP and DPP) may result from by three isoforms of BMP-1 activities [88 - 90]. TGF beta-1 downregulates DSPP expression (80A and 80B) In contrast, BMP-2 activates DSPP expression via NF-Y signaling (91).

DSPP KO mice display tooth alterations that are very similar to the human dentinogenesis imperfecta type III (91, 92).

5.2.3.1.1.1 DSP

Dentin sialoprotein (DSP) originates from the N-terminal part of DSPP. It is a less phosphorylated molecule, rich in aspartic acid, glutamic acid, glycine and serine, related to sialoproteins (9% sialic acid) With a molecular weight of 52.5 kDa, DSP contains about 350- amino acids and 75 monosaccharides (29.6% carbohydrate) DSP contributes ~ 5-8% of the NCP in dentin. Porcine DSP is a proteoglycan with GAG chains containing chondroitin 6-sulfate (93).

In vitro, in a gelatin gel diffusion system at low concentrations (>25 microg/ml), DSP slightly increases the yield of HA at 3.5 and 5 days. At a higher concentration (50-100 microg/ml), it inhibited accumulation where fewer mineral crystals form and that tend to aggregate. Relative to the other ECM proteins, DSP appears to be a less effective mineralization regulator, although it does inhibit calcium phosphate mineral crystal formation and growth (94).

In vivo: Immunostaining shows localization of DSPP in the dentinal tubules of the peritubular dentin. The phenotype of DPP conditional KO mice, in which DSP is expressed but the DPP portion is not, indicated restoration of deficits in dentin volume but not in the mineral density observed in DSP null mice. This suggests that DSP is mainly involved in regulating matrix formation, whereas DPP is involved in initiation and maturation of mineralization (95).

5.2.3.1.1.2. DGP

The middle portion of DSPP migrates at 19kDa on SDS-PAGE. Discovered in the pig dentin extract (87), it has not been determined whether the molecule is present in other species and consequently if it has any role in the process of dentinogenesis.

5.2.3.1.3 DPP, including Phosphophoryn (PPs)

DPP is a cleavage product from the C-terminal side of DSPP. Its molecular weight is about 100kDa in rats, bovine and porcine. In humans, DPP migrates around 140kDa. It is a highly acidic phosphoseringe-rich protein with a high content of aspartic acid. DPP accounts for more than 50% of the NCP in most dentin. DPP binds to calcium, HA and collagen, and induces intrafibrillar mineralization (96)

DPP displays a C-terminal domain coding region sequence (a 244 residue sequence) This region has been named DMP2 (dentin matrix protein 2), and this part is not phosphorylated. Thus it has a much lower calcium binding capacity than the whole length PP. PP folds into a compact globular structure, whereas DMP2 is maintained in an IDP.

In vitro, PP at fairly comparable concentrations nucleates plate-like apatite crystals, whereas DMP2 fails to induce the transformation of amorphous calcium phosphate into HA. The phosphate moieties of phoshoryn are the
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Dentinogensis is based on both molecule (99), proteins, however both the N-terminal and C-terminal domains are glycosylated with ~155 phosphates per molecule (99).

Our knowledge on the role of phosphophoryn in dentinogenesis is based on both in vivo and in vitro studies.

When the localization of Phosphophoryn was examined by immunostaining, light staining was observed over the odontoblasts and proximal processes. No staining is observed over predentin. Staining is seen in intertubular dentin. The same staining pattern is seen with “Stains All” (9) The staining is present in copious amounts at the mineralization front, suggesting that DPP is involved in the initiation of mineral crystal formation and in maturation of dentin minerals. As mentioned above, DPPc KO mice initiated mineral crystal formation and in maturation of mineralized dentin (95).

DPP causes nucleation at low concentrations, and inhibition of crystal growth at high concentrations (100, 101). Phosphophoryn is adsorbed preferentially on the (100) face of the large (non-physiologic) mineral crystals (102). This specific binding is one way in which DPP can regulate the size and shape of the mineral crystals.

To summarize this section, DPP or phosphophoryn appears as a candidate to be implicated in intertubular dentin mineralization. Taken together, the silver grains, immunolabeling and histochemical staining of dentin: structure, composition and mineralization are involved in maturation of mineralized dentin (95).

5.2.3.1.2. DMP-1

Dentin Matrix Protein-1 (DMP-1) is a highly phosphorylated acidic NCP that is potentially glycosylated. Pig DMP-1 has 510 amino acids, plus a 16 amino acid signal peptide. With a molecular weight of 53.5 kDa, DMP-1 possesses 93 serines and 12 threonines in the appropriate context for phosphorylation. The molecule is very similar to the Bone Acidic Glycoprotein (BAG75), although BAG75 is somehow heavier than the DMP1. In dentin extracts, DMP-1 appears as fragments with molecular weights between 30 and 45 kDa [103]. The full-length protein is cleaved into the C-terminal and the N-terminal fragments. This is due to the proteolytic cleavage by BMP-1/Tolloid-like proteinases into NH2-terminal sequence (104). There is also a GAG-glycosaminoglycan (chondroitin sulfate) containing fraction (105)

DMP-1 interacts with other molecules, regulates DSPP gene transcription (106) and is downregulated by TGF beta1. The molecule is present in odontoblasts, dentinal tubules and ameloblasts. DMP-1 is immunolocated predominantly at the mineralization front. Therefore DMP-1 is candidate to play a role in dentinogenesis, in addition to the role it plays in regard to its signaling properties.

In vitro: DMP-1 is involved in the regulation of biomineralization, which may be due to its calcium binding capacity. It also binds with great affinity to collagen fibrils. It has the capacity to induce heterogenous nucleation of calcium-phosphate crystals, and regulates crystal growth. DMP-1 immobilized on Type I collagen fibrils facilitates apatite deposition in vitro. The non-phosphorylated recombinant protein acts as a HA nucleator (107). Phosphorylated recombinant DMP-1 has no detectable effects, nor is it an inhibitor of HA formation and growth. Both the N-terminal and C-terminal fragments are HA nucleators, while the GAG-PG fragment is an effective inhibitor of HA formation and growth (108) The dephosphorylated forms of the fragments all have a less significant effect than the phosphorylated forms.

In vivo: Studies on DMP-1 deficient mice suggest that there is a direct effect of the molecule on mineral formation and crystal growth, and an indirect effect on the regulation of Ca x P concentration and matrix turnover (109).

Effects of mutation: DMP-1 mutation is important in an autosomal-recessive form of hypophosphatemic rickets. Regulation of phosphate homeostasis occurs through Fibroblast Growth Factor 23 (FGF23) (110) (Figure 16).

Deletion of DMP-1 induces the partial failure of maturation of predentin into dentin and hypomineralization, and causes the expansion of pulp and root canal cavities during post-natal development. The phenotype of the mice with mutations in DMP-1 is similar to dentinogenesis imperfecta (DI) type III, where DSPP is reduced. Thus, it appears that there may be an interaction between DSPP and DMP-1 via a regulation of DSPP by DMP-1 (111). Both in vitro and in vivo approaches substantiate the role of DMP-1 in circumpulpal intertubular formation.

5.2.3.1.3. BSP

Bone SialoProtein (BSP) is also a candidate for playing a role in the vertebrate biomineralization process. BSP constitutes ~1% of the total NCP in dentin (112). The molecule is post-translationally modified by glycosylation, phosphorylation and sulphatation. The molecular weight of this glycoprotein is 60-80 kDa, with high carbohydrate content reported to represent ~50% of the molecular weight. Therefore the protein core by itself has a molecular weight of 33-34kDa. BSP is characterized by the repetition of several polyglutamic acid segments and by an arginine-glycine-aspartate (RGD) motif that mediates cell attachment. It has been shown to promote the initial formation of mineral crystals in vitro, with the nucleational ability dependent on both the extent of phosphorylation and the domain that binds to the mineral surface (113) BSP is a marker of osteogenic differentiation, but there are relatively lesser amounts in dentin (114).

The role of BSP in biomineralization may be to mediate the initial stages of connective tissue mineralization, as reviewed in the following references (114, 115). However, it must be noted that the BSP
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knockout mouse has a variable, almost non-detectable bone phenotype, casting doubt on this hypothesis (116, 117).

A limited number of reports are focusing on the effects of BSP on reparative dentinogenesis (118). In contrast to bone, nothing is known on the role of BSP in "normal" or "physiological" dentin formation. Three intrinsic properties may be important with respect to dentinogenesis: BSP intensifies collagen fibrillation, promotes crystal nucleation and the RDG sequence favors/promotes adhesion of cells to the ECM.

5.2.3.2.1. Osteopontin OPN

dentinogenesis is not known. After briefly reviewing these molecules, we will focus on another series of potential molecular players in dentinogenesis.

5.2.3.2. Other dentin ECM proteins

5.2.3.2.1. Phosphorylated ECM molecules

The role of other dentin ECM molecules is more ambiguous, either because they are ubiquitous molecules, or because they act as mineralization inhibitors in many biological models. This is the case for osteopontin, MEPE and osteocalcin, and little is known about their functions in dentinogenesis except that they are present in the tissue extract, sometime as molecules intensively expressed by the odontoblasts, and sometimes detected after dentin immunolabeling. In addition, as it is the case for SPARC, they may act as calcium-binding proteins, but their role in dentinogenesis is not known. After briefly reviewing these molecules, we will focus on another series of potential molecular players in dentinogenesis.

5.2.3.2.1.1. Osteopontin OPN

Osteopontin is a ~ 34 kDa (314 amino acids) nascent protein in humans that is a variably phosphorylated sialoprotein, characterized by the presence of a polyaspartic acid sequence and sites of Ser/Thr phosphorylation that mediate HA binding. An RGD motif mediates cell attachment/signaling. OPN is not only a structural protein but also a matricellular molecule, implicated in inflammatory processes (119). The OPN null mice did not have a detectable dental phenotype, and no other data are yet forthcoming to define the role of the molecule in dentinogenesis.

In vitro: The polyaspartate-containing OPN is a mineralization inhibitor that depends on its status phosphorylation. Recombinant non-phosphorylated OPN and chemically dephosphorylated OPN have no effect on HA formation. Highly phosphorylated milk OPN promotes HA formation. It may be concluded that OPN phosphorylation is an important factor in the regulation of an OPN-induced mineralization process (120).

5.2.3.2.1.2. MEPE/OF45 (osteoregulin)

The Matrix Extracellular Phosphoglycoprotein (MEPE) has a central portion, including an RGD sequence, a glycosaminoglycan-attachment sequence (SGDG), and a putative calcium-binding motif. In bone, MEPE appears to be an inhibitor of mineralization as the MEPE null animal is hypermineralized. The acidic, serine- and aspartate-rich MEPE-associated motif (ASARM) is an effective mineralization inhibitor (Figure 15).

In vitro, the phosphorylated intact protein is an effective promoter of mineralization in the gelatin diffusion system. Dephosphorylated MEPE and dephosphorylated ASARM peptide have no effect on mineralization (121).

In vivo: The central portion of MEPE (Dentonin) is effective in promoting the differentiation of pulp cells into odontoblasts/osteoblast progenitors. Immunostaining using an anti-MEPE antibody stains the predentin, but not the dentin (122). The lack of staining of metadentin suggests that there is no influence of the molecule on intertubular dentin formation.

Effects of the mutation: The gene coding for MEPE is also located on the human chromosome 4q21. However, the mutation does not produce a dentinogenesis imperfecta or a dentin dysplasia, but its effect is pronounced on X-link hypophosphatemic rickets and causes the occurrence of large interglobular spaces in circumpulpal dentin, which are filled with ECM molecules accumulating in these spaces instead of diffusing in the whole dentin. No effects of the mutation are detectable in the mantle dentin, indicating that MEPE is not essential for this tissue's formation (123).

5.2.3.2.2. Other ECM molecules

5.2.3.2.2.1. Osteocalcin OCN

Some confusion arises between osteocalcin (BGP) and bone matrix gla protein (MGP). Only mineralized tissues synthesize BGP (OC), while MGP is synthesized by soft tissues, perhaps as an attempt to prevent their calcification. Treatment of rats with a vitamin K antagonist (e.g. warfarin) causes secretion of a non-gamma-carboxylated BGP that does not bind to HA, accumulates in bone and blocks ossification. OCN is an inhibitor of tissue transglutaminase activity, but is not a mineralization inhibitor.

Immunocytochemical localization of OCN in rat teeth shows more positive staining in mantle dentin, and far less staining in circumpulpal dentin (124).

In the OCN KO mice, the bone cortices are thickened relative to age-matched controls. Fourier Transformed Infra Red Microscopy (FT-IRM) suggests that OCN is required to stimulate bone mineral maturation or osteoclast remodeling. Reports in the literature suggest that locally produced levels of OCN are not sufficient to influence dentinogenesis (125). It is not clear, however, why a protein involved in remodeling and osteoclast action should be present in a tissue where there is little or no remodeling.

The Hyp mice model is a model for X-link hypophosphatemic rickets. Although MEPE should primarily be affected, intense expression of OCN has been reported, with similar circumpulpal dentin defects, as reported above (126).

Expression of OCN is high in differentiating odontoblasts, and even increased at later stages. Recent studies have suggested that OCN is involved in glucose metabolism (127), however, while it is possible that these cells have greater metabolic needs, it is not clear why OCN...
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would be localized in mature differentiating odontoblasts.

Matrix gla protein (MGP) is clearly a mineralization inhibitor in many biological models. Tissue specific knockout of MGP in teeth (128) had extensive hypomineralization, with amounts of unmineralized ECM from four- to eight-fold higher in dentin and alveolar bone when compared with that in wild-type tissues. Moreover, mineralization was absent in tooth root dentin and cellular cementum, while crown dentin showed "breakthrough" areas of mineralization. Acellular cementum was missing in these animals, and unmineralized odontoblasts formed within the pulp. Thus MGP is also important as an inhibitor of dentin mineralization.

5.2.3.2.2. Secreted Protein, Acidic and Rich in Cysteine (SPARC)

The Secreted Protein, Acidic and Rich in Cysteine (SPARC), is a basement membrane protein (BM-40) also known as osteonectin. This ubiquitous molecule is located at distinct sites in the many tissues where it is found. Its molecular weight is about 43 kDa in mice. The molecule consists of 300 amino acids, plus a signal peptide made up of 17 amino acids. Without post-translational modifications, the molecular mass of SPARC in mice is ~32 kDa. Three domains form the molecule: an acidic Ca2+-binding domain, a follistatin-like domain and an extracellular calcium-binding domain, as described (129).

SPARC mRNA is visualized by in situ hybridization in many soft and hard tissues, including odontoblasts in dentin (130). As a Ca2+- binding protein and a collagen-binding protein, SPARC may contribute indirectly to dentin mineralization, but this has yet to be confirmed.

5.2.3.2.2.3. Ca++-binding proteins and metallo-enzymes

Calmodulin and some members of the annexin family are also present both in odontoblasts and in forming dentin (131). Altogether these molecules may be implicated in Ca++ transport towards the extracellular matrix and the phosphoproteins therein, the role of these proteins in this mechanism has not been demonstrated. Annexin V has been shown to be involved in the nucleational complex of matrix vesicles, and thus may play some role, direct or indirect, in mineralization.

Other Ca++-binding proteins may also play roles in dentinogenesis; this has been suggested in the case of nucleobindin, a Ca2+ binding protein found in odontoblasts (nucleus, RER, mitochondria) and within the surrounding dentin ECM. It may contribute to the accumulation and transport of Ca2+ ions to the mineralization front (132).

Enzymes such as alkaline phosphatase, a Zn- and Mg-containing enzyme, and cation-binding proteins derived from the blood serum and other molecules listed in Table 3 have been identified in dentin extracts. Tissue non-specific alkaline phosphatase (TNAP) activity seems to be crucial in the dephosphorylation of some ECM proteins. This enzyme hydrolyzes pyrophosphate and provides inorganic phosphate to promote mineralization (133). Other enzymes also play a role in odontogenesis. The indirect effects of MMP-2, MMP-9 and MMP-3 inhibition are documented (134, 135). The two former enzymes are found in dentin, mostly at the dentino-enamel junction. The latter is present in predentin and plays a role in proteoglycan regulation (42). Thrombospondin 1, a protein whose activity is dependent on calcium (136) is expressed by odontoblasts and is also found in predentin (137), but the direct role of these molecules in dentinogenesis has not been elucidated.

In contrast, two groups of extensively studied molecules seem to play a crucial role in dentin formation and mineralization. Some data obtained in the laboratories of this article’s authors stimulate our interest for the potential role of the two groups of molecules, proteoglycans and lipids, in dentinogenesis.

5.2.4. Proteoglycans (PGs)

5.2.4.1. Small Leucine-rich proteoglycans (SLRPS)

Chemical analyses of dentin revealed that there are several SLRPs (Small Leucine-Rich Proteoglycans) present, and larger (aggregating) proteoglycans are also present, but in lesser amounts. Light and electron microscopy histochemical methods established the presence of glycosaminoglycans (GAGs) in predentin and dentin (39) (Figures 5, 16-18) The general concept developed initially is that GAGs are most abundant in predentin and barely detectable in dentin. The same was reported for bone. The conclusion drawn at that time was that GAGs are mineralization inhibitors and must be removed by cleavage, followed by subsequent degradation in the place where mineralization should be initiated. This was a matter of contention, because GAGs and PGs were identified in the mineralized compartment. Therefore they were clearly not destroyed, but were thought to be enzymatically modified. More recently it has been recognized that they may also represent a second group of PGs. Indeed radioautographic data evidence two distinct groups of GAGs in predentin and dentin. GAGs in predentin (PD-PGs) have a rapid turnover. They form an amorphous gel, allowing for the transportation/fibrillation of collagen fibrils moving from the proximal to the distal parts of the predentin. The presence of stromelysin-1 (MMP-3) explains how the PD-PGs can be degraded and why they turn over so rapidly (42). The second group of GAGs is secreted in dentin near the mineralization front (PGs) These PGs are stable, are incorporated into the forming dentin and become dentin components associated with mineralization (44).

Indeed gradients of distribution were found in predentin, with some CS/DS-containing GAGs destroyed or reduced in the distal predentin, whereas some KS-containing GAGs were more abundant in the distal third of the pre-dentin (40).

Histochemical, autometallographic, enzyme-gold labeling and ultrastructural studies show that PGs are actually present in dentin. They appear as "crystal-ghost” organic envelopes which persist after demineralization. The crystal ghosts contain a mixture of proteins, GAGs and phospholipids. The size of the
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Figure 17. A. Cuprolinic blue-aldehyde fixation allows the formation of large aggregates in predentin (pd) B and C reveal the presence of a substrate for hyaluronidase (GAGs) in predentin (pd) and dentin (d). Pretreatment of the section with hyaluronidase abolishes the enzyme gold labeling, demonstrating the specificity of the labeling.

Figure 18. Differences appear between biglycan (bgn) and decorin (dcn) immunolabeling in the molar (mol) and in the incisor (inc) aggregates is smaller than in predentin, but the number of aggregates is not reduced compared with predentin. Autometallography using cuprolinic blue as cationic dye shows a reinforced staining in metadentin, at the predentin-dentin edge (138).

Evidence for a potential role of PGs in dentin mineralization is supported by in vitro studies demonstrating the capacity of biglycan to initiate crystal nuclei [139], and by observations carried out in vivo on biglycan, decorin and fibromodulin KO mice (23,24).

Biglycan (BGN) and decorin (DCN) are two CS/DS SLRPs, coded by different genes but with very similar compositions. Some differences appear in predentin with respect to the diameter of collagen fibrils (enhanced in BGN-deficient mice, and apparently not influenced by DCN-deficiency) and the density of the fibrils (enhanced in DCN-deficient mice only) In both cases, the metadentin and dentin appear to be less mineralized than in the wild type control mice (23). This suggests that variations in the collagen fibril’s diameter may reduce the access of calcium phosphate to sites of mineralization on the collagen fibrils, by changing their intrinsic physico-chemical (structural, electric interactions) properties. It is also possible that the postponed modification of predentin components into dentin also delays the mineralization process. Indeed this was a striking feature in newborn and young mice, whereas in the adults such differences were diminished or abolished by what may be compensatory mechanisms (Figure 18).

The same features were detected in young fibromodulin (Fmod)-deficient mice (from newborn to 21-day-old mice) Dentinogenesis was impaired, and compensatory mechanisms then rescued mineralization impairment (24). Again, interactions (i.e., up- and down-regulation) of other ECM molecules may be occurring. When analyzing the phenotype of Dspp-/- Dcn -/-, the enlarged predentin found in the Dspp KO is rescued by the absence of DCN. This does not occur in the absence of BGN (Dspp-/-, Bgn-/-). Hypomineralization is similar in both cases, but the lack of BGN increases the number of calciospherites (140).

Interestingly, fibromodulin (Fmod) has a lower MW in mice dental tissue (40kDa) compared to bone (52kDa) in the same animals. In cartilage, MMP-13 cleaves fibromodulin, and therefore the difference between the bones and teeth may be due to either differential cleavage by MMP-13 or even from deglycosylation processes. In Fmod-deficient mice, the expression of DSPP is enhanced. As for DSPP, DMP-1 may be involved in compensatory mechanisms (i.e. is up-regulated), and in growing, but older, mice (~ 10 weeks old), such features become undetectable. A smaller diameter of collagen fibrils was found in the tail of Fmod-null mice. An uneven distribution of collagen fibrils was also reported in the periodontal ligament, accompanied by increased interfibrillar spaces (141) We have observed a reverse situation in predentin, wherein the collagen fibrils in the Fmod-deficient mice have an increased diameter, as was the case for BGN-KO mice (24). Dentin and mandibular bone hypomineralization was observed, together with a twisted appearance of enamel rods (142). Altogether, these data provide evidence that these PGs play a role in dentin formation and mineralization. This suggests a direct mechanism of PGs in dentinogenesis, but this effect may also be influenced by the binding of DCN and BGN to TGF beta, sequestering TGF beta reservoirs within the dentin matrix (143).

5.2.4.2. Large aggregation chondroitin/keratan sulphate family members in dentin: versican

Versican is a member of the large aggregating chondroitin/keratan sulphate family, also called hyaluronan-binding. This family includes aggrecan, versican, brevican and neurocan. Only versican has been identified as an intact molecule in the pulp, whereas in dentin it is a fragmented molecule. Immunostaining revealed moderate or weak labeling in the peritubular dentin (144).
**5.2.5. Phospholipids and proteolipids**

For years a sudanophilic band was reported to be present at the dentin-predentin junction at the dentin edge (145, 146). However, the method used was questionable because it required treatments of the tissue that would remove any lipid, and indeed all the methods available for light microscopic histochemistry so far have failed to confirm it’s the lipids at this localization. Indeed, it was demonstrated that the sudanophilic property is due to the presence of lipophic proteins, and not lipids (147). Using malachite green or iodoplatinate, we were able to stain lipids and observe their distribution by electron microscopy (19, 148) (Figure 19). Phospholipids, which may be removed chemically (chloroform/methanol or acetone) or enzymatically (phospholipase C) are located in the predentin of intercollagen spaces. Co. collagen. (B) Theses aggregates are suppressed by methanol pretreatment before mga staining, confirming the fact that phospholipids are stained with this method.

![Figure 19. mga staining identifies malachite green/aldehyde/phospholipids aggregates (A) (B) In the predentin (pd) but not in undemineralized dentin. In (C) after demineralization (dem), mga resistant material is seen in dentin (D) around collagen fibrils. After cryofixation (A), larger aggregates form a network in predentin, in intercollagen spaces. Co. collagen. (B) These aggregates are suppressed by methanol pretreatment before mga staining, confirming the fact that phospholipids are stained with this method.](image)

As these data ascertained the presence of phospholipids, but did not reveal a possible function nor suggest a mechanism, we also investigated genetic or genetically-induced diseases (Krabbe’s and Fabry’s disease) and pharmacologically-induced pathology (zinc deficiency, suramin- and chloroquine- induced lipidosis) (19). These models display accumulation of intracellular lipids in large lysosomes, but the dentin structure was close to normal, except for the presence of some large lipidic inclusions.

By studying the effects of the chemically-induced fro/fro (fragilitas ossium) mutation, documented to be a non-collagenous, severe form of osteogenesis imperfecta, we observed in the newborn and in the young mice (up to 21 days) several cellular and extracellular modifications, due to the mutation of the neutral sphingomyelin phosphodiesterase [151]. In the young mice, dentinogenesis was altered, and a type II dentin dysplasia was observed (152). This is the first time that a clear-cut relationship could be established between the alterations of a gene coding for an enzyme implicated in the degradation of some lipids located in the ECM. However, this lipid may have a role in signaling osteogenesis, so the detailed mechanism requires further investigation.

The interaction between [3H] procollagen and phospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine liposomes, is weak or null. In contrast, procollagen I binds strongly to sphingomyelin in a reversible and storabe manner. (130, 153). This may apply either to matrix vesicle mineralization. Changes in phospholipid composition accompany mineralization of chicken growth plate cartilage matrix vesicles. Sphingomyelin degradation may be a prerequisite for crystalline mineral formation (154).

At the mineralization front of bone, phospholipids were identified in calcospherulites (155). In atherosclerotic lesions, the proteoglycan decorin links low-density lipoproteins (LDL), sphingomyelin being a constituent of plasma lipoproteins, to collagen Type I (156). This points to a possible complex mechanism where small leucine-rich PGS, LDL phospholipids after cleavage by neutral sphingomyelinas, and collagen Type I interact to promote initial mineralization in dentin, or calcification at ectopic sites.

**6.CONCLUSIONS**

Dentin formation and mineralization provide an excellent model to study a variety of mineralization processes. Specifically, we suggest:

1) Matrix vesicles present at the onset of the outer dentin layers display a hierarchical distribution of membrane phospholipids, coated by proteoglycans, and

phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatic acid, and sphingomyelin (19).
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associated with calcium-binding proteins and enzymes. Together these anhydrous sites contribute to the initiation and growth of a mineral phase.

2) The formation of circum pulpal dentin occurs in a three-compartments model: odontoblast cell bodies, predentin and dentin. Native collagen and one group of PGs are implicated in collagen fibrillation and transport from the proximal border to the outer predentin-dentin junction. At that place, non-collagenous proteins are secreted: a second group of PGs, SIBLINGs, and other non-phosphorylated proteins. Taken together, they contribute to the formation of intertubular dentin. Some blood-serum-derived molecules take an intercellular pathway and also may contribute to dentin formation/mineralization. A mineralized phase is formed in dentin, appearing as needle-like structures at the surface and between collagen fibrils. The interaction between phosphoproteins, lipoproteins, and proteoglycans may coat the oriented crystals.

3) The formation of peritubular dentin results from intercellular diffusion. Albumin, alpha-2 HS glycoprotein, choline-rich phospholipids contribute to the formation of a highly mineralized dense ring reinforcing the tubule, where there is no collagen. A phospholipids-proteolipid complex may be formed in the absence of collagen (15-18).

4) Other dentin-like structures, reactionary or reparative dentins, are similar to events driving bone formation, except that there is no dentinoclasts or remodeling effects.

Interactions between ECM molecules, the role of specific domains exposed after cleavage by proteases or matricryptic events, the occurrence of isoforms and the folding of ECM molecules, pave the way for a better understanding of, and therefore an enhanced ability to mimic, the process of mineralization.

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