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Dentin: Structure, Composition and Mineralization:

The role of dentin ECM in dentin formation and mineralization

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I-Introduction

A thick dentin layer forms the bulk of dental 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 in their central parts dental pulps, which 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.

II- II- Dentin : structure and ultrastructure- the three-compartments model

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

Phylogenetic studies have revealed that during evolution, originally dentin analogues were very similar to bone, with osteoblast/odontoblast-like cells located within alveoli, as it 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. Odontoblasts 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** 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. The diameter of tubules varies between 2 and 4 micrometers. The number of dentine tubules is about 18 000 and 21 000 tubules per mm2 [5]. They are more numerous in the inner third layer than the outer third layer of the dentin.

II-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, having few thin and curved tubules. . In the root, similar layers are identified as follows 1) a **Tomes granular layer,** formed by calcospheritic 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 hypothesis may be drawn from their physical and chemical -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 dentin-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 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 produces within the circumpulpal dentin enlarged non-mineralized interglobular spaces containing dentin ECM molecules [12]. Hence, the nature of the NCP’s differs between the mantle and circumpulpal dentins, the outer layer being not influenced by the organic phosphorous status. As a consequence, in the crown, the outer layers are less mineralized than the rest of the dentin. They display some elastic properties and therefore provide some resilience, important from a mechanical point of view and allowing dissipation of stress forces [7]. However, the thickness of the outer layer is about 200mm, therefore larger than the presumed width of the mantle dentin.

In the root of the tooth, again the elastic modulus is 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 mantle dentin, and lacking in many cases. Bent and narrow tubules are deviated by calcospheritic structures present in the granular Tomes’s layer.

At this early stage, it worth to note that polarizing odontoblasts parallel with the basement membrane display some buddings. These membrane protrusions detach and become matrix vesicles very similar to what has been described in cartilage and bone, and these vesicles are thought to contribute to the initial dentin mineralization [14]. This is the only instance where 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.

II-2-Circumpulpal dentins

The circumpulpal dentin forms the largest part of the dentin layer. Thin at initial stages of dentinogenesis, its thickness continuously increases (about 4mm/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,. The ratio between inter-tubular and peri-tubular dentin is highly species dependant. 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, s form the plate-like crystallites, 2–5 nm in thickness and 60nm in length. At lowermagnification, inter-tubular crystallites have a needle like appearance. They are located either at the surface the collagen fibrils, and parallel withthe collagen fibril axis; the crystallites randomly fill interfibrillar spaces [19]. Demineralization of intertubular dentin reveals a dense network of collagen fibrils, coated by non-collagenous proteins. Glycosaminoglycans as 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 higher resolution, the crystals are bearing the following measurements: a= 36nm, b=25nm and c= 9.75nm [21]. 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].

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 10 micrometers thick layer, reduced afterward to a daily 4 micrometers deposit. 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 peri-tubular dentin** results from two different type 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 the synthesis of extracellular matrix molecules (ECM) components along with a long process where 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 ECM molecules, namely collagen and proteoglycans, 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 proteoglycans (decorin, biglycan, lumican, fibromodulin) implicated in collagen fibrillation [23, 24]. Some non-collagenous proteins (NCP) 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 20nm, whereas in the central part the mean diameter reaches 40nm, and in the distal part, near the mineralization front, fibril diameter vary between 55–75nm [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, three are 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 a processes shared by bone and dentin. However, while there may be some similarities between bone and tooth formation it is also clear there they also have unique properties. For example, bone formation is followed by a constant remodeling due to osteoclast-osteoblast interactions, hormonal influences and matrix metalloproteinase (MMP)s degradation of the existing matrix proteins, whereas after its formation, dentin is a quite 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 matrix, may be secreted by the odontoblast processes within dentin, or taking 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 so far studied, 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, beneath a calciotraumatic line, interpreted as an interruption of normal dentinogenesis, **reactionary** or **tertiary dentin** is formed. This dentin is relatively? unstained by the “stains all” method, hence is deficient in acidic proteins some of which are presumed to be phosphorylated. As iss the case for the mantle dentin, this is due either to a defective post-translational modification (ie phosphorylation), or to the absence of these proteins. Reactionary dentin appears either as a layer of the osteodentin type, or as a tubular or atubular orthodentin, depending 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 the release of some components of dental material fillings, free-monomers of resins or silver amalgam containing mercury. Reactionary dentin is synthesized by odontoblasts, or if these cells are altered, this layer is produced by the subjacent cells of the Höehl‘s layer, issued for the last division of pre-odontoblasts, which are latent adult progenitors. Reactionary dentin is different from what is named **reparative dentin**. However, this last type of so-called “dentin” does not result from the activity of odontoblasts or their associated cells, but specifically from pulp progenitors, implicated in the formation of a bone-like or in structure-less mineralization (pulp diffuse mineralization or pulp stones). Such structures are closer to bone rather than to dentin.

To conclude this part, 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, type I collagen playing a major role, at least as 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 are producing specifically very different tissues. The unanswered question is why is it necessary to have three totally different processes involved in the formation of dentin in a single tooth. This leads to the

question of What are the determinants for selecting one of the three pathways or why one over other?

III- Odontoblasts: implication in the synthesis, secretion and mineralization of dentin ECM

III-1. Odontoblasts and dentin formation

III-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 in the median region, odontoblasts precursors migrate from the fronto-nasal bud. Interaction 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 allows 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].

III-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 cilium, 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 species, tight junctions. These junctional complexes constitute a solid permeability membrane, and intercellular diffusions are restricted to molecules with small molecular weight. 4) Fenestred 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].

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: rough endoplasmic reticulum, Golgi apparatus, immature and mature secretory vesicles, associated with lysosomal equipment (GERL, small and large lysosomal vesicles, multivesicular structures), and ii) a long process protruding in the predentin and adhering to the dentinal walls of the tubules. The question of the length of the process remains unanswered. Formerly, it was assumed that the processes reach the dentin-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 extended more than half distance between the pulp and the DEJ. Immunocytochemical data reveal that cytoskeletal proteins are found up to the DEJ, hence that the process may extend up to that interface. It is however possible that the processes withdraw but some non-functional remnants of the process may remain, adhering to the tubule wall [31].

III-1-3-The functional odontoblasts: synthesis and secretion of extracellular matrix components

III-1-3-1-Collagen: Radioautographic data using labeled amino acids such as ^{3}H proline, have clarified the mechanisms of incorporation and the fate of this major collagen constituent, 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 and 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]

After 24 hours, the labeling is very weak in odontoblasts, firmly reduced in predentin and then only an accumulation of silver grains is seen forming a dense band 10–20 micrometers in widthat 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.

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. 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. Radioautographic data and experiments using microtubule inhibitors suggest the occurrence of 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].

III-1-3-2-Phosphorylated proteins: Using [^{3}H] serine and [^{33}P] phosphate as phosphoprotein precursors, Weinstock & Leblond [36] showed in the rat that after 30 min silver grains are seen over odontoblasts cell bodies, in the Golgi region. The major difference with [^{3}H 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 [^{33}P] 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, at 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 show both stainings at the mineralization front. In both cases the

staining is missing after prior pretreatment of the sections with alkaline phosphatase [37, 38]. This substantiates the occurrence of a 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.

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 CS/DS antibody (2B6) also reveals intensive staining in predentin [40, 41]. In addition, the occurrence of gradients of distribution between CS/DS and 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 subsequent coiling during the shrinkage due to dehydration, the GAGs appear as stellar or boomerang-like structures located along the collagen fibrils or as in close association with the so-called holes due to the quarter stagger structure of the fibrils [39].

III-1-3-3-Glycosaminoglycans and proteoglycans: In this context, after [^{35}S] sulfate injection, a time course study revealed a dual localization of the labeling [44]. One part rapidly incorporates into the predentin, moving eventually 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 [^{35}S] 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. [^{3}H] 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, [^{3}H] serine and [^{35}S] 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 or not 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 [^{3}H] fucose labeling, at least for the fucose-containing glycoproteins, slightly diverging results having been published. Four hours are necessary before silver grains accumulation 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].

III-2-Intercellular 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 where the incorporation of some ECM components may follow intercellular pathways independently from any cell control. When [^{3}H] choline was injected intraveinously, 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 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 membrane 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 (tights and gap junctions) may account for the difference in the diffusion process [52]. Albumin is considered as 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, ^{45}Ca directly reaching the mineralization front at the dentin edge within 30 sec to 5 min after injection, without 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 of the labeled precursor, secretion either occurs in the proximal predentin or at the distal predentin- inner dentin edge. The two different sites are related respectively 1- to the release of collagen fibrils and their associated proteoglycans in predentin, or 2- to the discharge of non-collagenous phosphorylated proteins and mineral associated proteoglycans that are secreted at the mineralization front or metadentin [22]. Some matrix components migrate directly from the serum to the dentin compartment. They follow mainly an intercellular pathway, albumin and phospholipids being implicated in the transport of mineral toward and therefore in the mineralization process of intertubular dentin.

IV- 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 into the formation and mineralization of dentin. They may act either as promoter or as inhibitor depending on conocentration 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, 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, OPN and BSP are identified as matricellular proteins, but others molecules seem to play similar role. ECM molecules may also be implicated into **cell signaling** [58]. These three properties interact and make a classification difficult.

IV-1- Mechanisms of dentin mineralization: How ECM molecules contribute to the formation of a mineral phase

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 and among many examples includes ectopic calcification, vascular calcification and kidney stone formation. Calcification may occur as a result from hypercalcemia, or maybe induced by post-necrotic events. Spontaneous precipitations of mineral complexes may also occur, but in general not in an apatitic form. Often, terminology is not always used in a proper way. Calcification is usually a non-physiologic event, mineralization is generally physiologic. Biomineralization, in general, is the process through which cells orchestrate the deposition of mineral. 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) the cell derived-matrix vesicles driven mineralization occurring mainly in the mantle dentin, 2) the ECM molecules-derived mineralization, accounting for the majority of dentin formation, and 3) The 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 proposing a general concept about 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. They have been reported in the literature as “crystal ghosts”. These structures combine proteins, proteoglycans and phospholipids, and they have been identified as phospho- glyco- lipo- 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 the formation of the mantle dentin in teeth and 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 [Kirsch T, Wang W, Pfander D. Functional differences between growth plate apoptotic bodies and matrix vesicles. J Bone Miner Res. 2003 Oct;18(10):1872-81.]] 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-dependant. Fibronectin, and then collagen are deposited. Non-collagenous proteins are post-translationally modified by casein kinases (phosphorylation), protein phosphatases (dephosphorylation), 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. [Qin C, D'Souza R, Feng JQ. Dentin matrix protein 1 (DMP1): new and important roles for biomineralization and phosphate homeostasis. J Dent Res. 2007 Dec; 86(12):1134-41.

IV-2-ECM molecules implicated in dentinogenesis

There have been a variety of attempts to identify the proteins and other matrix components implicated in dentinogensis. These range from protein [ref] to gene expression studies[ ], to gene discovery studies defining the dentinogenesis transcriptome [Simon S, Smith AJ, Lumley PJ, Berdal A, Smith G, Finney S, Cooper PR. Molecular characterization of young and mature odontoblasts. Bone. 2009 Oct;45(4):693–703.].

IV-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 decorin [63]. Osteoadherin, PHEX, DSPP, BSP and enamelysin are also expressed in rat(?) dentin [64].

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 theso-called SIBLING proteins, the non-phosphorylated non-collagenous proteins (e.g. osteocalcin) and for some members of the 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 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, 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 specific domains of the molecule, which are exposed or hidden in some presentations of the molecule Many of the ECM proteins are intrinsically disordered [Tompa P. Structure and Function of Intrinsically Disordered Proteins, CRC Press, Boca Raton, FLA, 2009], having flexible structures that vary as they interact with their miscellaneous partners (other proteins, enzymes, mineral). These IDPs include osteopontin and bone sialoprotein [Fisher LW,

Torchia DA, Fohr B, Young MF, Fedarko NS. Flexible structures of SIBLING proteins, bone sialoprotein, and osteopontin. Biochem Biophys Res Commun. 2001 Jan 19;280(2): 460-5.; Huq NL, Cross KJ, Ung M, Reynolds EC. A review of protein structure and gene organisation for proteins associated with mineralised tissue and calcium phosphate stabilisation encoded on human chromosome 4. Arch Oral Biol. 2005 Jul;50(7):599–609.]., DMP1 [Gericke A, Qin C, Sun Y, Redfern R, Redfern D, Fujimoto Y, Taleb H, Butler WT, Boskey AL. Different forms of DMP1 play distinct roles in mineralization. J Dent Res. 2010 Apr;89(4):355-9.] and amelogenin [Delak K, Harcup C, Lakshminarayanan R, Sun Z, Fan Y, Moradian-Oldak J, Evans JS. The tooth enamel protein, porcine amelogenin, is an intrinsically disordered protein with an extended molecular configuration in the monomeric form. Biochemistry. 2009 Mar 17;48(10):2272-81.], In light of the concept of cryptic sites and IDPs the role of matricryptic sites has been reported [65]. 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.

IV-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 [ ]. Type I collagen and results from the self-assembly of two alpha1(I) chains and 1 alpha 2 (I) chain.. These chains are assembled in a triple helix with a coiled coil conformation [66]

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 [67]. Collagen fibrillogenesis is influenced by dentin small leucine-rich proteoglycans [68]. Removal of GAGs delays fibrillogensis. PGs inhibit fibrillogenesis and fibrils thickness over concentrations 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 [69–70]. It was shown that the abacus-like structures are acid phosphatase-containing structures, and therefore lysosomes [71]. 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[72].
- 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 newly 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% in the distal third. This suggests that the fibrils are pushed toward the place where mineralization occurs. Flux of forces are 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].

- The transfer of collagen fibrils within the predentin from the proximal to the distal parts assumes that an amorphous ground substance plays role serving as a viscous gel allowing the fibrils 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].
- The mean diameters of the collagen fibrils in mice molars are enlarged in the BGN- and fibromodulin-deficient mice, whereas no difference is detected between the wild type and decorin-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 it 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 also 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 [73].
- Altogether, it is clear that collagen fibrils are important in dentinogensis mostly by providing 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 [74]

IV-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 crucial role in dentinogenesis [56].

IV-2-3-1-SIBLINGs: They have been initially found in mineralized tissues (bone and teeth), but they have also be identified in soft tissues such as salivary glands, kidney and prostate tumors.

In dentin, the SIBLING family includes DSPP, immediately cleaved after secretion into DSP, DGP and DPP. It also contains DMP-1, BSP, osteopontin and 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 reveal the importance of the SIBLINGs in dentinogenesis. The distribution of SIBLINGs varies when analyzed by the 3 steps mode of extraction [75]:

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, 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 (NCP) 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 EDTA solution procedure, followed by guanidium-HCl treatment. Only BSP is present in the G2 extract.

In addition, an association between some SIBLINGs and 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 [76, 77]. With regards to dentinogenesis, such partnerships may impact the cleavage of these structural molecules and subsequently, expose new biologically active domains.

IV- 2-3-1-1-DSPP: The Dentin SialoPhosphoProtein is expressed mostly in dentin, but not exclusively. The molecule has been detected in bone at only 1/400th the level as 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 [78]:

1. the Dentin Sialo Protein (DSP) – the N-terminal region of DSPP, with a Mw of 155kDa, with a total length of 360–370 amino acids- aa 16-374),
2. the Dentin Glycoprotein (DGP) (Mw around 19kDa(amino acids 375–462) [79],
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-1activities [80].

TGF beta-1 downregulates DSPP expression (80A and 80B)In contrast, BMP-2 activates DSPP expression via NF-Y signaling [81].

DSPP KO mice display tooth alterations that are very similar to the human *dentinogenesis imperfecta* type III [82].

DSP: Dentin sialoprotein 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 [83].

In vitro, in a gelatin gel diffusion system at low concentrations (>25 microg/ml) DSP slightly increase the yield of HA at 3.5 and 5 days. At 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 [84].)

In vivo: Immunostaining shows localization of DSPP in the dentinal tubules in the peritubular dentin. The phenotype of DPP conditional KO mice in which DSP is expressed but not the DPP portion indicated restoration of deficits in dentin volume but not in the mineral density observed in DSPP null mice suggesting that DSP is mainly involved in regulatingmatrix formation whereas DPP is involved in initiation and maturation of mineralization [85].

DGP: The middle portion of DSPP migrates at 19kDa on SDS-PAGE. Discovered in the pig dentin extract [79], 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.

DPP, including Phosphophoryn (PPs): DPP is a cleavage product from the C-terminal side of DSPP. Its molecular weight is about 100kDa in rat, bovine and porcine. In humans, DPP migrates around 140kDa. It is a highly acidic phosphoserine-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 and HA, binds to collagen and induces intrafibrillar mineralization [.Cocking-Johnson D, Sauk JJ. The interaction of bovine dentine phosphophoryn and collagen during fibrillogenesis of collagen in vitro. Biochim Biophys Acta. 1983 Jan 12;742(1):49–53.

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 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 phosphoryn are the mediators of mineralization [86] since dephosphorylated DPP has no effect on in vitro mineralization [Milan AM, Sugars RV, Embery G, Waddington RJ. Adsorption and interactions of dentine phosphoprotein with hydroxyapatite and collagen. Eur J Oral Sci. 2006 Jun;114(3):223-31.]. The N-terminal region of DPP is distinct from other ECM proteins, however both. the N-terminal and C-terminal domains are glycosylated, with ~155 phosphates per molecule. Our knowledge on the role of phosphophoryn in dentinogensis is based on both in vivo and in vitro studies.

In vivo studies: When the localization of Phosphophoryn was examined by immunostaining,, light staining is 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 amount at the mineralization front suggesting that DPP is involved in the initiation of mineral crystal formation. and in maturation of dentin mineral. As mentioned above, DPPcKO mice showed restored the dentin volume but not the mineral density observed in DSPP KO mice. This suggested DPP is more involved in maturation of mineralized dentin 85].

- DPP causes nucleation at low concentrations, and inhibition of crystal growth at high concentrations [89, 90]. Phosphophoryn is adsorbed preferentially on the (100) face of the large (non-physiologic) mineral crystals [91] . 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 candidate to be implicated in intertubular dentin mineralization. Taken together, the silver grains, immunolabeling and histochemical staining of metadentin, at the dentin edge near the predentin, support this assumption.

IV-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 [92]. 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 [93]. There is also a GAG-glycosamino glycan (chondroitin sulfate) containing fraction [Qin C, Huang B, Wygant JN, McIntyre BW, McDonald CH, Cook RG, Butler WT. A chondroitin sulfate chain attached to the bone dentin matrix protein 1 NH2-terminal fragment. J Biol Chem. 2006 Mar 24;281(12):8034-40]

DMP-1 interacts with other molecules, regulates DSPP gene transcription [94] 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 role in dentinogenesis, in addition to its signaling properties.

In vitro:DMP-1 is involved in the regulation of biomineralization and may do so by the fact that it has 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 1 collagen fibrils facilitates apatite deposition *in vitro*. The non-phosphorylated recombinant protein acts as a HA nucleator. Phosphorylated recombinant DMP-1 has no detectable effects or is 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 [Gericke A, Qin C, Sun Y, Redfern R, Redfern D, Fujimoto Y, Taleb H, Butler WT, Boskey AL. Different forms of DMP1 play distinct roles in mineralization. J Dent Res. 2010 Apr;89(4): 355-9.] The dephosphorylated forms of the fragments all have a less significant effect. than the phosphorylated formed In vivo:Studies on DMP-1 deficient mice suggest that there is a direct effect of the molecule on mineral formation, crystal growth and an indirect effect on the regulation of Ca x P concentration and matrix turnover [96].

Effects of mutation: DMP-1 mutation is important in an autosomal-recessive form of hypophosphatemic rickets. Regulation of phosphate homeostasis occurs through FGF23 [97].

Deletion of DMP-1 induces the partial failure of maturation of predentin into dentin, 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, there it appears that there may be an interaction between DSPP and DMP-1 via a regulation of DSPP by DMP-1. Both in vitro and in vivo approaches substantiate the role of DMP-1 in circumpulpal intertubular formation.

IV-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 [99]. 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 a 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 both on the extent of phosphorylation and the domain that binds to the mineral surface[Baht GS, O'Young J, Borovina A, Chen H, Tye CE, Karttunen M, Lajoie GA, Hunter GK, Goldberg HA. Phosphorylation of Ser136 is critical for potent bone sialoprotein-mediated nucleation of hydroxyapatite crystals. Biochem J. 2010 Apr 9. [Epub ahead of print].]

BSP is a marker of osteogenic differentiation, but there are relatively lesser amounts in dentin [Chen S, Chen L, Jahangiri A, Chen B, Wu Y, Chuang HH, Qin C, MacDougall M. Expression and processing of small integri -binding **ligand** N-linked glycoproteins in mouse odontoblastic cells. Arch Oral Biol. 2008 Sep;53(9):879-89.]..

The role of BSP in biomineralization may be to mediate the initial stages of connective tissue mineralization, as reviewed in the following references [100, 101]. However, it must be noted that the BSP knockout mouse has a variable, almost non-detectable bone phenotype, casting doubt on this hypothesis [Malaval L, Wade-Guéye NM, Boudiffa M, Fei J, Zirngibl R, Chen F, Laroche N, Roux JP, Burt-Pichat B, Duboeuf F, Boivin G, Jurdic P, Lafage-Proust MH, Amédée J, Vico L, Rossant J, Aubin JE. Bone sialoprotein plays a functional role in bone formation and osteoclastogenesis. J Exp Med. 2008 May 12;205(5): 1145-53.]

A limited number of reports are focusing on the effects of BSP on reparative dentinogenesis [refs]. In contrast with bone, nothing is known on the role of BSP on "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.

IV-2-3-2-Other dentin ECM proteins

IV-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, osteocalcin, and little is known on their function in dentinogenesis, except that they are present in the tissue extract, sometime as molecules intensively expressed by the odontoblasts, and sometimes they are 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 candidate players in dentinogenesis.

IV-2-3-2-1-1-Osteopontin OPN: Osteopoontin is a ~ 34 kDa (314 amino acid) nascent protein in humans, that is a variably phosphorylated sialoprotein, characterized by the presence of polyaspartic acid sequence and sites of Ser/Thr phosphorylation that mediate HA binding. A RGD motif mediates cell attachment/signaling. OPN is not only a structural protein but also a matricellular molecule, implicated in inflammatory processes [102]. The OPN null mice did not have a detectable dental phenotype, and no other data are yet forthcoming to define a role of the molecule in dentinogenesis.

In vitro: The polyaspartate-containing OPN extracted from one 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 OPN-induced mineralization process [103].

IV-2-3-2-1-2- MEPE/OF45 (osteoregulin): The Matrix Extracellular Phosphoglycoprotein (MEPE) has a central portion including a 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 e mineralization inhibitor. 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 [104]. In vivo: The central portion of MEPE (Dentoin) 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. 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 *dentinogensis 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, filled with ECM molecules accumulating in these spaces instead of diffusing in the whole dentin. No effect of the mutation is detectable in the mantle dentin indicating that MEPE is not essential for this tissues' formation [105].

IV-2-3-2-2-Other ECM molecules

IV-2-3-2-2-1- Osteocalcin OCN: Some confusion arises between osteocalcin and bone matrix gla protein (BGP). Only mineralized tissues synthesize BGP (OC) while MGP is synthesized by soft tissues, perhaps as an attempt to prevent their calcirication. . 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 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 [106].

In the OCN KO mice the bone cortices are thickened relative to age-matched controls. . r Fourier Transformed Infra Red Microscopy (FT-IRM) suggests that OCN is r equired 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 [107]. It is not clear, however why a protein involved in remodeling and osteoclast action should be present in a tissue where there is li or nottle 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 [108].

Expression of OCN is high in differentiating odontoblasts, and even increased at later stages. Recent studies [Ferron M, Hinoi E, Karsenty G, Ducy P. Osteocalcin differentially regulates beta cell and adipocyte gene expression and affects the development of metabolic diseases in wild-type mice. Proc Natl Acad Sci U S A. 2008 Apr 1;105(13):5266-70.] have suggested that OCN is involved in glucose metabolism, however it is not clear why it would be localized in mature differentiating odontoblasts, although it is possible that these cells have greater metabolic needs. **Matrix gla protein (MGP)** is clearly a mineralization inhibitor in many biological models. Tissue specific knockout of MGP in teeth [Kaipatur NR, Murshed M, McKee MD. Matrix Gla protein inhibition of tooth mineralization. J Dent Res. 2008 Sep; 87(9):839-44.] 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. More over, 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 osteodentin formed within the pulp. Thus MGP is also important as an inhibitor of dentin mineralization.

IV-2-3-2-2-2- SPARC: The Secreted Protein, Acidic and Rich in Cysteine (SPARC), is as a basement membrane protein (BM-40) alsoknown as osteonectin. This ubiquitous molecule is located at distinct sites in the many tissues where it is found. Its molecular weight is about 38 and 35 kDa in ???? (SPECIES). The molecule consists of 300 amino acids, plus a 17amino acids signal peptide. Without post-translational modifications the molecular mass of SPARC in ??? (SPECIES) is ~32 kDa. Three domains form the molecule: an acidic Ca2+-binding domain, a follistatin-like and an extracellular calcium-binding domains have been described [109].

SPARC mRNA is visualized by in situ hybridization in many soft and hard tissues, including odontoblasts in dentin [110]. As a Ca2++ binding protein, and a collagen-binding protein, SPARC may contribute indirectly to dentin mineralization, but this has to be confirmed.

IV-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 [111]. 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 protein may also play roles in dentinogenesis: thishas 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 [112].

Enzymes such as **alkaline phosphatase**, a Zn- and Mg- containing enzyme, and cation biinding 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 [113].. Other enzymes also play a role in odontogenesis. The indirect effects of MMP- 2, MMP-9 and MMP-3 inhibition are documented [114, 115]. The two former enzymes are found in dentin mostly at the dentino-enamel junction. The later is present in predentin and plays a role in proteoglycan regulation [42]. **Thrombospondin 1**, a protein whose activityu is dependent on calcium [Calzada MJ, Kuznetsova SA, Sipes JM, Rodrigues RG, Cashel JA, Annis DS, Mosher DF, Roberts DD. Calcium indirectly regulates immunochemical reactivity and functional activities of the N-domain of thrombospondin-1. Matrix Biol. 2008 May;27(4):339-51.] is expressed by odontoblasts and also found in predentin [Ueno A, Yamashita K, Nagata T, Tsurumi C, Miwa Y, Kitamura S, Inoue H. cDNA cloning of bovine thrombospondin 1 and its expression in odontoblasts and predentin. Biochim Biophys Acta. 1998 Jan 15;1382(1):17–22], but the direct role of these molecules in dentinogenesis has not been elucidated.

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

IV-2-4-Proteoglycans (PGs)

IV-2-4-1-Small Leucine-rich proteoglycans (SLRPS): Chemical analyzes of dentin revealed there are several **SLRP**s (Small Leucine-Rich Proteoglycans), and larger (aggregating) proteoglycans present but in lesser amount. Light and electron microscopy histochemical methods established the presence of glycosaminoglycans (GAGs) in predentin and dentin [39]. 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 at place where mineralization should be initiated. This was matter of contention, because GAGs or PGs were identified in the mineralized compartment. Therefore they were clearly not destroyed, but were thought to be enzymatically modified. More reently 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 turn-over. They form an amorphous gel allowing the transportation/fibrillation of collagen fibrils moving from the proximal to the distal part of the predentin. The presence of stromelysin-1 (MMP-3) explains how the PD-PGs can be degraded and why they turnover so rapidly [42]. The second group of GAGs is secreted in dentin near the mineralization front (PGs). These PGs are stable, 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 are more abundant in the distal third of the pre-dentin [40].

Histochemical, autometallographic, enzyme-gold labeling and ultrastructural studies showPGs 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 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 [116].

Evidences for a potential role of PGs in dentin mineralization are supported by in vitro studies demonstrating the capacity of biglycan to initiate crystal nuclei [117] 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 composition. 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 appears to be less mineralized than in the wild type control mice [23]. This suggest that variations in collagen fibrils diameter may reduce the access of calcium phosphate to sites of mneralization 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 adult such differences were diminished or abolished by what may be compensatory mechanisms.

The same features were detected in young fibromodulin (Fmod) -deficient mice (newborn and up to 21 day-old mice). Dentinogenesis was impaired, and compensatory mechanisms then rescued mineralization impairment [24]. Again, interactions (ie, 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-/0). Hypomineralization is similar in both case, but the lack of BGN increases the number of calcospherites [118].

Interestingly, fibromodulin (fmod) has a lower MW in what species??? dental tissue (40kDa) compared to bone (52kDa) in the same animals. In cartilage MMP-13 cleaves fibromodulin thus difference between bone and teeth may result from 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 (ie is up-regulated), and in growing, but older mice (~ 10 weeks old), such features become undetectable. 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 inter fibrillar spaces [ref???]. We have observed a reverse situation in predentin, where the collagen fibrils in the Fmod- deficient mice have an increased diameter, as it was the case for BGN-KO mice [24]. Dentin and mandibular bone hypomineralization was observed, together with a twisted appearance of enamel rods [119]. 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 alsobe influenced by the binding of DCN and BGN to TGF beta, sequestering TGF beta reservoirs within the dentin matrix [120].

IV-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 named 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 [121].

IV-2-5-Phospholipids and proteolipids—For years a sudanophilic band was reported to be present at the dentin-predentin junction, at the dentin edge [122, 123]. However, the method used was questionable because it required treatments of the tissue that would remove any lipid, and indeed all the method so far available for light microscopyic histochemistry failed to confirm it’s the lipids at this localization. Indeed, it was demonstrated that the sudanophilic property is due to the presence of lipophilic proteins and not lipids [124]. Using malachite green or iodoplatinate we were able to stain lipids and observe their distribution by electron microscopy [19, 125]. Phospholipids, that may be removed chemically (chloroform/methanol or acetone) or enzymatically (phospholipase C) are located in predentin in inter-collagenic spaces, forming a continuous network. In dentin, needle-like structures or what has been named crystal ghosts forming a thin organic envelopes wrapping crystallites were stained, but no staining was seen at the mineralization front. Large amorphous dots of phospholipids/malachite green/aldehyde complexes are also stained within dentin.

This finding was corroborated by dot-blot analyses [126], and chemical analysis [127], showing that some acidic phospholipids are actually present during the formation of dentin. Because two thirds of the dentin lipids may be extracted prior to any demineralization procedure, the first extract displaying a composition close to membrane lipids is issued from odontoblasts cell bodies and processes. The remaining third is extractible only after demineralization. Therefore, it was assumed that these acidic phospholipids are specifically associated to the mineralization phase. The lipid composition of the second extract is formed by phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatic acid, and sphingomyelin [19].

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 studing 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 young mice (up to 21 days), cellular and extracellular modifications due to the mutation of the neutral sphingomyelin phosphodiesterase [128]. In the young mice, dentinogenesis was altered, and a type II *dentin dysplasia* was observed [129]. 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 that the dtailed mechanism requires further investigation.

The interaction between [3H] procollagen and phospholipids such as phosphatidylcholine, phosphatidylethanoamine, phosphatidylinositol and phosphatidylserine liposomes, is weak or null. In contrast procollagen I bind strongly to sphingomyelin in a reversible and storable manner. [130]. This may apply either to matrix vesicle mineralization. Changes in phospholipid composition accompany mineralization of chicken growth plate cartilage matrix vesicles. Sphingomyelin degradation maybe a prerequisite for crystalline mineral formation [131].

At the mineralization front of bone, , phospholipids were identified in calcospherulites [different ref], [132]. In atherosclerotic lesions the proteoglycan decorin links low-density lipoproteins (LDL), sphingomyelin being a constituent of plasma lipoproteins, to collagen Type I [133]. This points to a possible complex mechanism where small leucine-rich PGs, LDL phospholipids after cleavage by neutral sphingomyelinase, and together with collagen Type I interact to promote initial mineralization in dentin, or calcification at ectopic sites.

To conclude, dentin formation and mineralization provides an excellent model to study a variety of mineralization process. 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 associated to calcium-binding proteins and enzymes. Together these anhydrous sites contribute to the initiation and growth of a mineral phase.
2. The formation of circumpulpal 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, proteoglycans may coat the oriented crystals.
3. The formation of peritubular dentin result 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 and 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, and therefore mimicking of the process of mineralization.

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Table 1

Global composition of dentin

Mineral phase	70% in weight	40–45% in vol
Organic matrix	20% in weight	30% in vol
Water	10% in weight	20–25% in vol

Table 2

Percentage of silver grains in odontoblasts, predentin and dentin [25]

	30 min	2h	4h
odontoblasts	70.5%	36.5%	27.0%
predentin	29.5%	63.5%	73.0%

Table 3

Global composition of dentin extracellular matrix [ECM] molecules

Collagens 90%		**Type I Collagen (89%) + Type I trimer (11%)**	**+ 1–3% Type III and V Collagens**
Non-Collagenous Proteins (NCP) # 10%	Phosphorylated proteins	SIBLINGs (Small Integrin-Binding LIgand, N-linked Glycoproteins) [56]	**DSPP (Dentin Sialo Phospho Protein)** (Mw between 155 et 95kDa), after cleavage : • **DSP (Dentin sialo Protein)** (N-terminal-proteoglycan forming dimers) : 100–280kDa • **DGP (Dentin Glyco Protein)** : 19kDa • **DPP (Dentin Phospho Protein or dentin phosphoryn)** (C-terminal) 94kDa mineralization nucleator **DMP-1 (Dentin Matrix Protein-1)** : 61kDa, a proteoglycan, nucleator **BSP (Bone SialoProtein):** 95kDa, proteoglycan, nucleation & crystal growth. **OPN (Osteopontin)** : 44kDa glycoprotein (mineralization inhibitor). **MEPE (Matrix Extracellular Phospho glyco Protein)** 66kDa, glycoprotein (mineralization inhibitor)
	=	SLRPs (Small Leucine-Rich Proteoglycans)	Decorin (and possibly other SLRPs)
	=	Amelogenin	Forms resulting from alternative splicing: • A+4 : 8.1kDa • A−4 (LRAP): 6.9kDa
	=	Other enamel protein	Ameloblastin
		Proteolipids	Matrix phospholipids
	Non-phosphorylated proteins	*Osteocalcin & DPG : dentin gla-protein (acid gamma carboxy glutamic-rich protein) * Matrix Gla Protein (MGP)	• 5.7kDa mineralization inhibitor • 14kDa, not a mineralization inhibitor
	=	Osteonectin or SPARC protein	43kDa
	=	Proteins taking origin from blood serum	*Albumin (lipid carrier) *Alpha$_2$-HS glycoprotein (only found in mineralized tissues) *Fetuin, implicated in serum-derived mineralization (nucleator HAp)
	=	SLRPS	CS/DS PGs: decorin, biglycan 42kDa KS PGs: lumican, fibromodulin, osteoadherin 50kDa
	=	Growth factors	FGF2, TGF beta1, BMPs, ILGF I & II, PDGF
	=	Enzymes	Alkaline and acid phosphatases, serine proteases Collagenases: MMP-1, -8, -13 Gelatinases: A: MMP-2, B: MMP-9 Stromelysin 1: MMP-3 MT1-MMP, enamelysine or MMP-20 ADAMs and ADAMTS Thrombospondin1
		Polyamines	Spermine, spermidine, putresceine
		Calcium binding proteins	Calmodulin, Calbindin, Annexines, Nucleobindin