Role of EMMPRIN and MMPs in tooth development, dental caries and pulp-dentin regeneration

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UNIVERSITE PARIS DESCARTES

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Laboratoire EA2496
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Pour obtenir le grade de
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Dirigée par Professeur **Catherine CHAUSSAIN**

Présentée et soutenue publiquement le 24 novembre 2014
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Dr. **ROCHEFORT Gael**, Université Paris Descartes	Examinateur
Dr. **HUET Eric**, Université Paris est Créteil	invité

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Table of Contents

Table of figures

List of abbreviations

AI: *Amelogenesis imperfecta*

Ambn : Ameloblastin

AMTN : Amelotin

Asn : Asparagine

ATK: serine/threonine kinase and is known as protein kinase B (PKB) or RAC-PK ('related to A and C protein kinase')

ASARM: acidic serine and aspartate-rich motif

BM: Basement membrane

BSP : Bone sialoprotein

BV : Bone volume

CyPA : Cyclophilin A

DI : *Dentinogenesis imperfecta*

DMP-1 : Dentin matrix protein 1

DSPP : Dentin sialophosphoprotein

DEJ: Dental enamel junction

ECM : Extra cellular matrix

Enam : Enamelin

EMMPRIN : Extra cellular matrix metalloproteinase inducer

GAG: Glycoaminoglycans

GIC: Glass ionomer cement

HAP : Hydroxyapatite crystals

HIF-1α : hypoxia-inducible factor 1α

KLK-4 : Kallikrine-4

KO: Knock out

MAPK: Mitogen-activated protein

MCT : Monocarboxylate transporter

MEPE: Matrix extracellular phosphoglycoprotein

MMP : Matrix metalloproteinase

MMP-1 : Collagenase 1

MMP-2 : Gelatinase A

MMP-3 : Stromelysin 1

MMP-9 : Gelatinase B

MMP-20 : Enamelysin

MVs : Membrane vesicules

NCPs: Non-collagenous proteins

OPN : Osteopontin

PDL : Periodontal ligament

PFA: Para-formaldehyde

PG: Proteoglycans

PI3K: Phosphoinositide 3-kinase

RGD: Arginine–glycine–aspartate cell adhesion domain

RA: Rheumatoid arthritis

SEM: Scanning electron microscopy

SIBLINGs : Small integrin-binding ligand N-linked glycoprotein

SLRPs: Small leucine-rich proteoglycans

Tb: Trabecular bone

TEM: Transmission electron microscopy

TIMP: Tissue inhibitor of MMPs

TV: Tissue volume

VEGF: Vascular endothelial growth factor

VOI : Volume of interest

General introduction and specific objectives

Tooth development results from reciprocal inductive interactions between the ectomesenchyme and oral epithelium and proceeds through a series of well-defined stages including the initiation, bud, cap and bell stages (Ruch, Karcher-Djuricic, and Gerber 1973; Slavkin 1974; Catón and Tucker 2009; Miletich and Sharpe 2003; I Thesleff and Hurmerinta 1981; Mitsiadis and Luder 2011). At the bell stage which is the last step of tooth crown formation, signals from the dental epithelium (i.e., inner enamel epithelium) instruct dental mesenchymal cells to differentiate into odontoblasts. Differentiated odontoblasts signal back to inner enamel epithelial cells and induce their differentiation into ameloblasts, which are responsible for enamel matrix synthesis. Ameloblast terminal differentiation necessitates the presence of an extracellular matrix that is secreted by odontoblasts and forms the predentin (Zeichner-David et al. 1995). The degradation of the basement membrane (BM) separating the dental epithelium from the mesenchyme is a key step in this process that allows direct contact of ameloblasts with both odontoblasts and the unmineralized dentin matrix (Catón and Tucker 2009; Olive and Ruch 1982). Matrix metalloproteinases (MMPs) are involved in all stages of tooth formation (Bourd-Boittin et al. 2005; Chaussain-Miller et al. 2006). At the bell stage, MMPs have a major role in BM degradation (Heikinheimo and Salo 1995; Sahlberg et al. 1992a), thus allowing direct cross-talk between odontoblasts and ameloblasts (Heikinheimo and Salo 1995; Sahlberg et al. 1999). It has been shown that at more advanced stages MMPs also regulate the processing of dental extracellular matrix (ECM) proteins prior to mineralization. Indeed, it has been demonstrated that MMPs regulate amelogenin (AMEL) cleavage by enamelysin (MMP-20) during early enamel maturation (Bourd-Boittin et al. 2005; Bourd-Boittin et al. 2004; Lu et al. 2008; Nagano et al. 2009; Turk et al. 2006; Simmer and Hu 2002; J. D. Bartlett and Simmer 1999). The notion of direct epithelial-mesenchymal (or epithelio-stromal) interactions was first introduced in the cancer field when EMMPRIN, a membrane glycoprotein also known as CD147, was identified as a MMP inducer present at the cell surface of tumor cells which can activate stromal cells through direct contact and signal them to increase MMP production (Toole 2003). Recently accumulating data also advocate a role for EMMPRIN in modulating MMP expression during non-tumorigenic pathological conditions as well as in physiological situations such as tissue remodeling and cytodifferentiation events (Gabison, Hoang-Xuan, et al. 2005; Huet, Gabison, et al. 2008; Mohamed et al. 2011; Kato et al. 2011; Nabeshima et al. 2006; L. Liu et al. 2010; Gabison et

al. 2009; Zhu et al. 2014) . The expression of EMMPRIN in the developing tooth germs was previously described (Schwab et al. 2007; Xie et al. 2010). EMMPRIN expression was shown to increase gradually in the forming molar germ in mice from E14 to P1(Xie et al. 2010). However, the *in vivo* role of EMMPRIN in tooth development and homeostasis is still unknown. **In this PhD, our first specific objective was to investigate EMMPRIN functions in tooth formation using EMMPRIN KO mice** by exploring the modifications occurring in their dental phenotype and the consequences on EMMPRIN's molecular targets, in particular on MMPs.

In parallel, EMMPRIN has been shown to be involved in the repair process of different injured tissues. Indeed, the role of EMMPRIN in wound healing through MMP induction and increase in myofibroblast contractile activity has been established (Gabison, Mourah, et al. 2005; Huet, Vallée, et al. 2008). As our team has developed several pulp injury models to follow-up the repair process, and as we had access to EMMPRIN KO mice it was tempting to study the repair process in this model. **Therefore, our second specific objective was to investigate for a potential role of EMMPRIN in the pulp dentin repair process by comparing the healing of injured pulps of EMMPRIN KO and WT mice.**

MMPs were shown to be expressed during tooth development and to be necessary for normal dentin formation (Bourd-Boittin et al. 2005). After dentin mineralization, they remain trapped in the calcified matrix either under active or proenzyme forms (Palosaari et al. 2003), which may explain their persistent presence within the dentin of adult teeth (A Mazzoni 2007; Tjäderhane et al. 1998). The role of these trapped MMPs in the progression of the carious process within dentin matrix has been proposed by several studies (Tjäderhane et al. 1998; Sulkala et al. 2001). Indeed, MMPs have been proposed to have an important role in the dentin organic matrix degradation following demineralization by bacterial acids (Tjäderhane et al. 1998; Chaussain-Miller et al. 2006). Cariogenic bacteria are essential to initiate the carious process but they cannot degrade the dentin organic matrix. After dissolution of the mineral part, the organic part of dentin becomes exposed to degradation by host-derived enzymes, including salivary and dentinal MMPs, and cysteine cathepsins (Nascimento et al. 2011; van Strijp et al. 2003). Because MMPs have been suggested to contribute to dentin caries progression, the hypothesis that MMP inhibition would affect dentin caries progression is appealing. This hypothesis was supported by in vivo studies in rat caries models where dentin caries progression was delayed by intra-oral administration of chemical MMP inhibitors, modified tetracylines and zoledronate (Sulkala et al. 2001;

Tjäderhane et al. 1999). Several natural molecules have been previously reported to have MMP inhibitory properties. Grape-seed extracts (GSE) have been shown to suppress lipopolysaccharide-induced MMP secretion by macrophages and to inhibit MMP-1 and MMP-9 activities in periodontitis (La et al. 2009). The MMP-inhibitory effects of these natural substances suggest, therefore, that they could be effective in inhibiting dentin caries progression. Recently, a new daily mouthrinse composed of grape-seed extracts and amine fluoride has been developed. **As grape-seed extracts are known to be natural inhibitors of MMPs, our last specific objective was to evaluate the capacity of these natural agents to prevent the degradation of demineralized dentin matrix by MMP-3.**

1 Introduction

1.1 Tooth description

Tooth is the hardest organ of the mammalian body and it provides several functions such as mastication, and phonation.

Anatomically, tooth structure can be distinguished in a visible part (crown) and a hidden part embedded in the alveolar bone of the jaw (root) (**Figure 1**). Instead of a considerably different shape and size (e.g., an incisor compared with a molar), teeth are histologically similar.

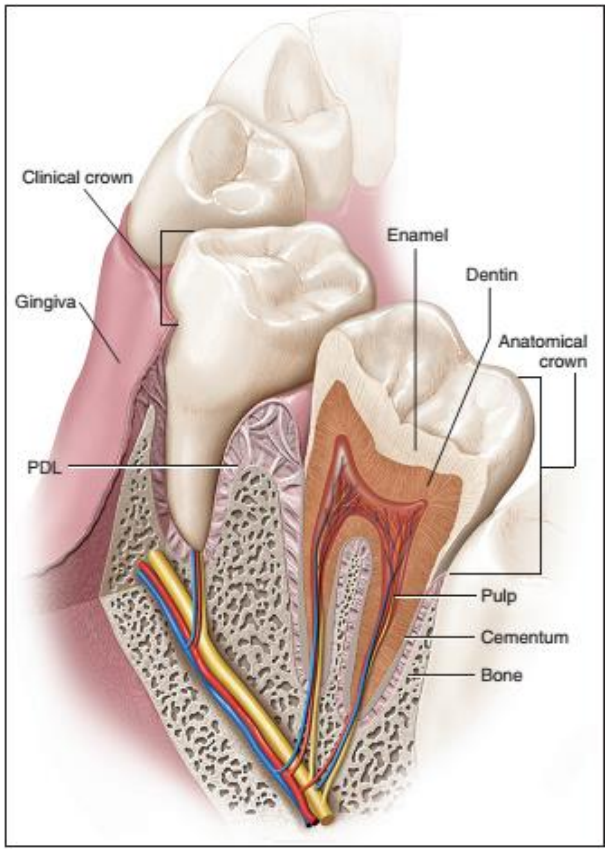

Figure 1 : **The tooth and its supporting structure. Adapted from** *(Antonio Nanci and Cate 2013)*

Tooth consists of several layers: enamel, dentine, cement, and dental pulp. The enamel is a hard, and acellular structure formed by epithelial cells and supported by dentin. This less mineralized, more resilient, and vital hard connective tissue, is formed and supported by the dental pulp, a soft connective tissue (**Figure 1**).

In mammals, teeth are attached to the bones of the jaw by the periodontium, consisting of the cementum, periodontal ligament (PDL) and alveolar bone, which provide an attachment with enough flexibility to withstand the forces of mastication.

Human and most of the mammals have two generations of teeth, primary and permanent; since the size of teeth cannot increase after formation, the primary dentition becomes inadequate and must be replaced by more and larger teeth (permanent dentition).

Otherwise, mice have only one generation highly reduced dentition having one incisor, separated from three molars by an edentulous region in each semi-maxilla (**Figure 2**.A). Incisor growth is continuous throughout the animal's life (**Figure 2**.B).

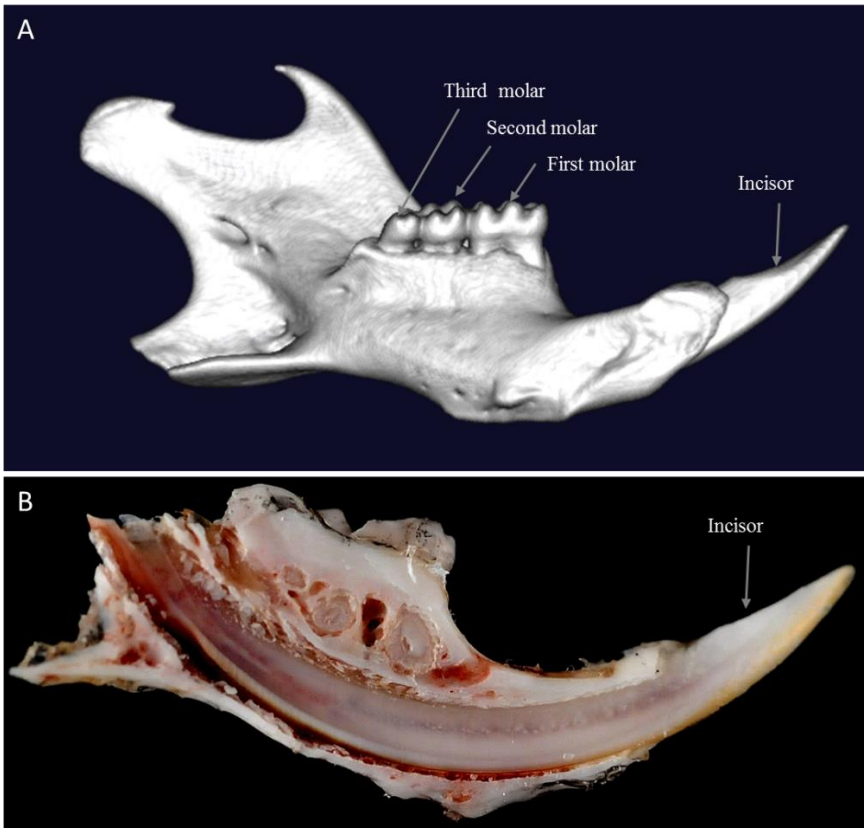

Figure 2 : **Adult mouse mandible (own data).**

1.2 Tooth development

Since toothed vertebrate have conserved tooth development process stages, data obtained from rodents studies may provide a lot of information about dental development in diverse species, including humans (Streelman et al. 2003).

Organogenesis results from three fundamental processes: I) initiation, within positional information are provided and interpreted to initiate organ formation at the right place; II)

morphogenesis, in which cells build up a rudimental organ; finally, III) differentiation where cells form organ-specific structures.

As also showed in mouse tooth development model (Irma Thesleff and Nieminen 1996), teeth are vertebrate-specific structures which, like many other organs, develop through a series of reciprocal interactions between two adjacent tissues, an epithelium and a mesenchyme (I Thesleff, Vaahtokari, and Partanen 1995). Tissue-recombination experiments have shown that the oral epithelium isolated from the mandibular arch of a mouse embryo, between embryonic day 9.0 and 11.5 (E9.0–E11.5), can stimulate a non-dental neural crest-derived mesenchyme to form a tooth. After E11.5, the odontogenic potential subsequently shifts from the epithelium to the mesenchyme, which can induce dental formation when combined with a non-dental epithelium, whereas the dental epithelium has lost this ability(Mina and Kollar 1987; Lumsden 1988).

1.2.1 Stages of tooth development

Tooth development takes place through a series of well-defined stages: epithelial thickening of the dental lamina, bud, cap and bell.

1.2.1.1 Dental lamina Stage

The thickening of the mouse oral epithelium is first visible at around E11.5 (**Figure 3**). The epithelial thickening forms the dental and vestibular lamina on the lingual and buccal aspect, respectively. The vestibular lamina forms a sulcus between the cheek and the teeth, and the dental lamina gives rise to the teeth. During this stage, dental lamina expresses several important signaling molecules such as (Sonic Hedgehog) Shh that increases cell proliferation at the tooth development site (Hardcastle et al. 1998).

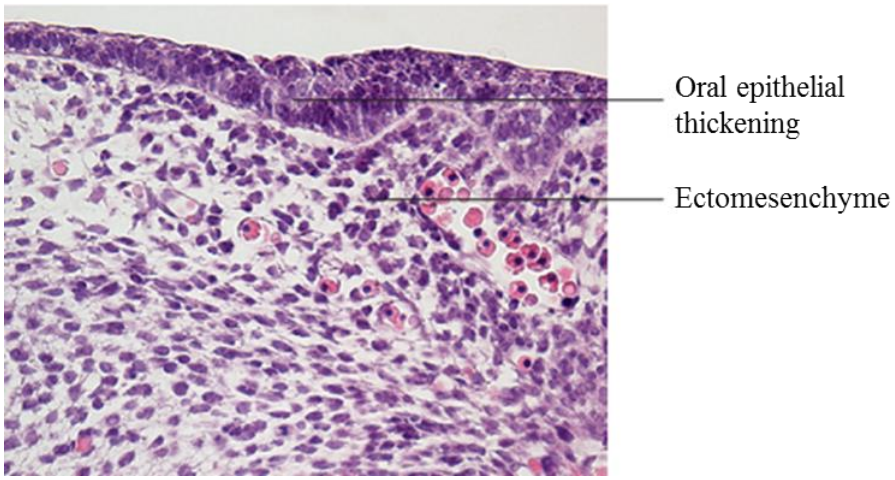

Figure 3 : **Dental lamina of tooth development. Adapted from** *(Antonio Nanci and Cate 2013)*

1.2.1.2 Bud stage

After the dental lamina stage, an epithelial structure that has a bud shape results from proliferating and invagination of the epithelium within the underlying ectomesenchyme. The bud is clearly formed at E13.5 and it consists in several layers: the dental follicle made by condensed mesenchymal cells, oriented in a radial pattern and encasing the dental papilla and the enamel organ; enamel organ, in which the internal epithelial cells meets the external epithelial cells and form a structure called the cervical loop; finally, dental papilla, which is a ball of densely packed ectomesenchyme (**Figure 4**).

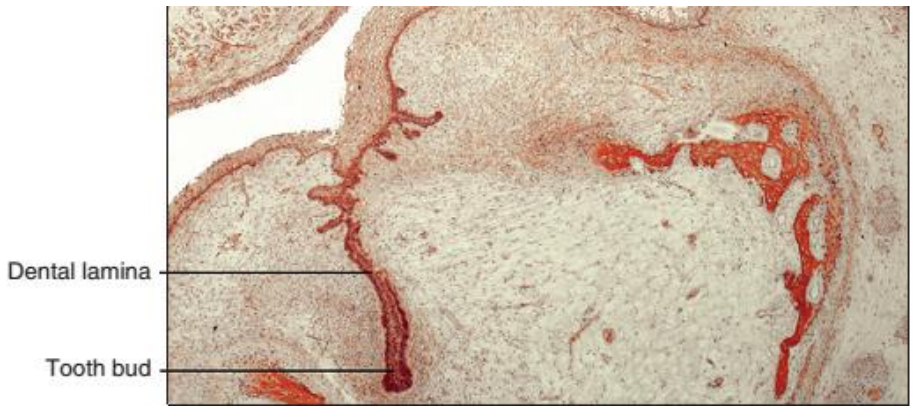

Figure 4 : **Bud stage of tooth development. Adapted from** *(Antonio Nanci and Cate 2013)*

1.2.1.3 Cap stage

Around E14.5, the condensing mesenchyme signals back to the enamel organ and induces the formation of a specific group of signaling epithelial cells known as the enamel knot which takes control of odontogenesis processes (Irma Thesleff, Keranen, and Jernvall 2001). The enamel knot is visible as a bulge in the center of the inner enamel epithelium at the cap stage (**Figure 5**). Enamel knot expresses a host of signaling molecules, such as Shh, Fgf4, Bmp4 and Wnt10b (Vaahtokari et al. 1996; Sarkar and Sharpe 1999).

Then, in multi-cusped teeth, secondary enamel knots guides the differentiation at each cusp tip, during the bell and crown formation stages (Irma Thesleff, Keranen, and Jernvall 2001; Matalova et al. 2005).

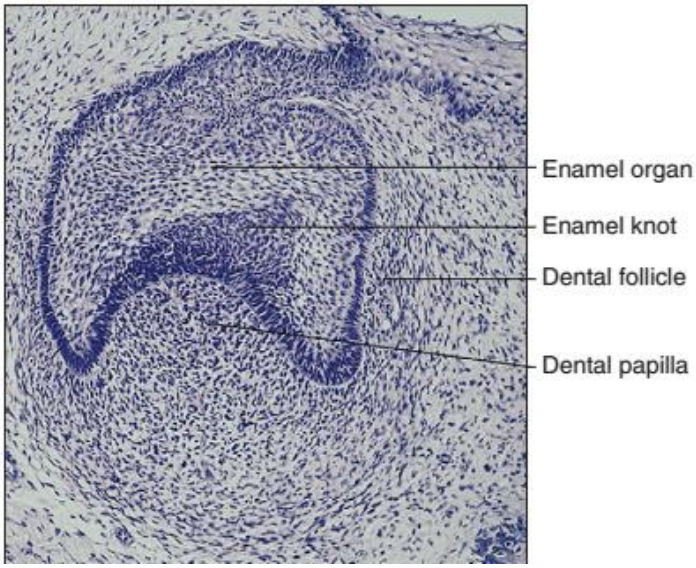

Figure 5 : **Cap stage tooth germ showing the position of the enamel knot. Adapted from** *(Antonio Nanci and Cate 2013)*

By E 15, the differentiation of enamel organ central cells forms the stellate reticulum cells (**Figure 6**) having a star shape with large intercellular spaces potentially playing a role in enamel-secreting ameloblasts nutrition. Another layer of cells known as "*stratum intermedium*", at E16.0 in the incisor and E17.0 in the molar, becomes recognizable from the internal dental epithelial cells as flattened epithelial cells, between the *stellate reticuIum* and the internal dental epithelium whose cells, progressively, lengthened to become preameloblasts.

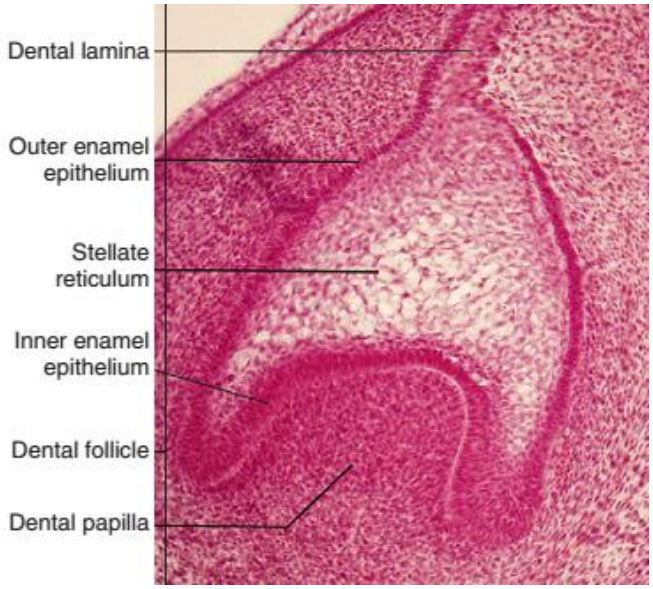

Figure 6 : **Cap stage, beginning of cellular differentiation within the enamel organ. Central cells form the *stellate reticulum*. Adapted from** *(Antonio Nanci and Cate 2013)*

1.2.1.4 Bell stage

During this stage (EI7.0 for incisor and by E17.5-18.0 for molars), dental papilla cells differentiate into odontoblasts, beginning in the most anterior mesenchymal cells (**Figure 7**). The external dental epithelial cells thickness decreases and becomes a one or two cuboidal cell layer.

The preameloblasts about double in height and differentiate into ameloblasts and their nuclei peripherally placed, this differentiation firstly occurs in the most anterior regions. The lingual side of the incisors does not become coated with enamel because that the internal dental epithelial cells do not differentiate into ameloblasts on this side. At El7.0 these non-differentiating internal dental epithelial cells, diminish and become cuboidal in shape in subsequent stages of development, then merge with adjacent connective tissue cells.

By EI8 in the incisors and El9 in molars, odontoblasts begin to secrete predentin (**Figure 7**). After 24 hours of development, the predentin starts mineralizing and enamel matrix will be secreted by ameloblasts. Mineralization of the enamel matrix is postnatal and the incisors and the first molar erupt by day 20 after birth (P20). Tooth shape will be established when mineralization of dentin and enamel starts.

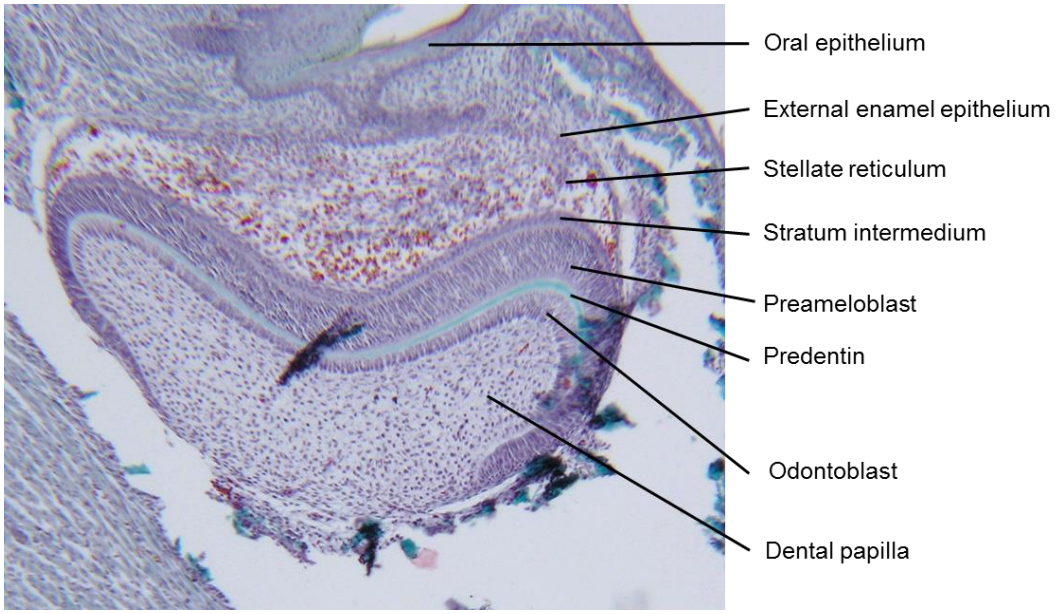

Figure 7 : **Early bell stage of tooth development (own data)**

1.2.1.5 Second and Third Molar Development

When the jaws elongate enough, the second and third molars start developing. Second molar development starts with the dental lamina, which can be seen at E15.5 forming as an outgrowth of the first molar tooth germ epithelium. By E18.5 the second molar is at the cap stage and erupts approximately at P25. The lamina of the third molar appears at P4, reaches the cap stage by P7-9 and the bell stage by P10, the third molar erupts by P35 (Rossant and Tam 2002).

1.2.2 Basement membrane

The basement membranes (BM) are the first extracellular matrices to appear and they are critical for organ formation and tissue repair (Martin and Timpl 1987; Kleinman et al. 1986). They act like scaffolds for cells and play an essential role in morphogenesis that affects cell adhesion, migration, proliferation, and differentiation.

The structure and components of BMs vary among tissues, resulting in tissue-specific structures and functions. BMs consist of supramolecular structure which is formed by reciprocal interaction of collagen IV, laminin, perlecan, nidogen/entactin, and other molecules (Martin and Timpl 1987; Kleinman et al. 1986).

BM components play an important role in tooth development. They control proliferation, polarity, attachment and determine tooth germ size and morphology (I. Thesleff et al. 1981; Fukumoto and Yamada 2005; Fukumoto et al. 2006).

For example, laminin α5 (Lama5), is a component of the major laminin chain in tooth basement membranes. Absence of Lama5 in KO mice lead to a small tooth germ with no cusps, in which the inner dental epithelium is not polarized and enamel knot formation is defective (Fukumoto et al. 2006).

Another laminins such as laminin α2 (Lama2) are expressed in odontoblasts during the late stage of germ development (Yuasa et al. 2004; Salmivirta, Sorokin, and Ekblom 1997). Its deficiency in mice manifests in thin dentin and defective dentinal tube structure (Yuasa et al. 2004). These phenotypes are similar to *dentinogenesis imperfecta* (DI) in humans. It was found that laminin-2, increases dentin sialoprotein expression in odontoblasts in cell culture, but its deficiency in mutant mice, reduces dentin sialoprotein expression in odontoblasts, suggesting that Lama2 is required for odontoblast differentiation.

Perlecan (HSPG2) is a major heparan sulfate proteoglycan in BMs. Its expression in developing teeth, was detected in BMs, intercellular spaces of the enamel organ, and the dental papilla including odontoblasts (Ida-Yonemochi et al. 2005). Overexpression of perlecan in transgenic mice results in abnormal tooth morphology and deregulation of growth factors such as TGF-b1 and bFGF (Ida-Yonemochi et al. 2011).

1.2.3 Dentin

1.2.3.1 Dentin structure

Dentin has a complex structure, similar to bone for mineralization ratio of about 70% mineral. In contrast with bone, dentin is not vascularized, and has not remodeling process. During the secretory stage, 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 stay outside the mineralized dentin, along the border of the mineralization front and the processes occupy the lumen of dentin tubules. Tubule diameter varies between 2 and 4 micrometers and its number is about 18 000 and 21 000 tubules per mm^2 (Schilke et al. 2000). They are more numerous in the inner third layer than the outer third layer of the dentin.

1.2.3.1.1 Outer mantel dentin layer

Outer mantel dentin is a thin atubular layer with thickness of 15–30µm, at the periphery of coronal region. It is less mineralized than the rest of dentin and consequently the resilient mantle dentin allow dentin to dissipate pressures which otherwise would induce enamel fissures and detachment of the fragmented enamel from the dentin-enamel junction(R. Z. Wang and Weiner 1998).

1.2.3.1.2 Circumpulpal dentins

The circumpulpal dentin appears as a thin layer at initial stages of dentinogenesis, its thickness continuously increases at the expense of the pulp and then it becomes the largest part of the dentin layer. The circumpulpal dentin is formed by circles of peritubular dentin around the lumen of the tubules separated by the intertubular dentin. The ratio between inter and peritubular dentin is species dependent, it is about 50% in horses and about 10-20% in humans, and in the continuously growing rodent incisors no peritubular dentin can be found.

Several differences in the structure and composition of these two types of dentin are found. In the intertubular dentin, the major protein is type I collagen (90%), whereas in the peritubular dentin no collagen is observed. The differences in the composition of noncollagenous proteins (NCPs) of the two types of dentin have been reported. (M. Goldberg, Molon Noblot, and Septier 1980; Weiner et al. 1999; Gotliv, Robach, and Veis 2006; Gotliv and Veis 2007).

Intertubular dentin (**Figure 9**) results from transformation of predentin into dentin (**Figure 8**). It is compound of dense network of collagen fibrils, coated by NCPs, where needle like-crystallites locate at the collagen fibrils parallel to their axes and other crystallites fill inter-fibrillar spaces (M. Goldberg and Boskey 1996).

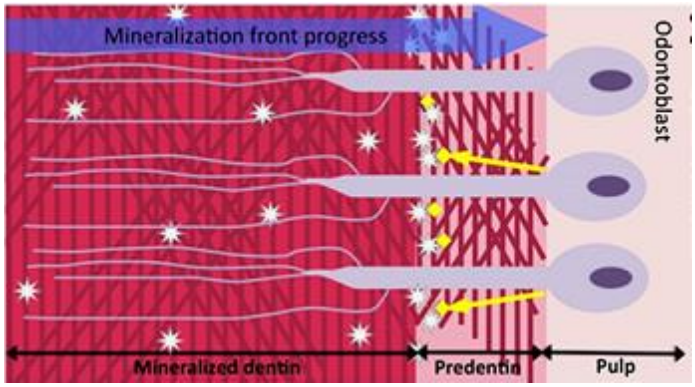

Figure 8 : **Characteristics of dentin formation. Odontoblasts secrete an ECM composed of type I collagen and NCPs. Within the predentin type I collagen molecules are assembled as fibrils. Mineralization occurs at the mineralization front by growth and fusion of calcospherites formed by hydroxyapatite (HAP) crystals. This mineralization process is controlled by NCPs and by mineral ion availability. Cell processes remain entrapped within dentin whereas cell bodies remain at the periphery of the pulp. Adapted from** *(Vital et al. 2012)*

Peritubular dentin result from a passive deposit of serum-derived molecules along the tubule walls and the crystals form a ring around the tubules lumen (**Figure 9**). In this type of dentin no collagen fibrils are detectable, but a thin network of non-collagenous proteins and phospholipids are visible (M. Goldberg, Molon Noblot, and Septier 1980; Gotliv and Veis 2007; M. Goldberg and Boskey 1996).

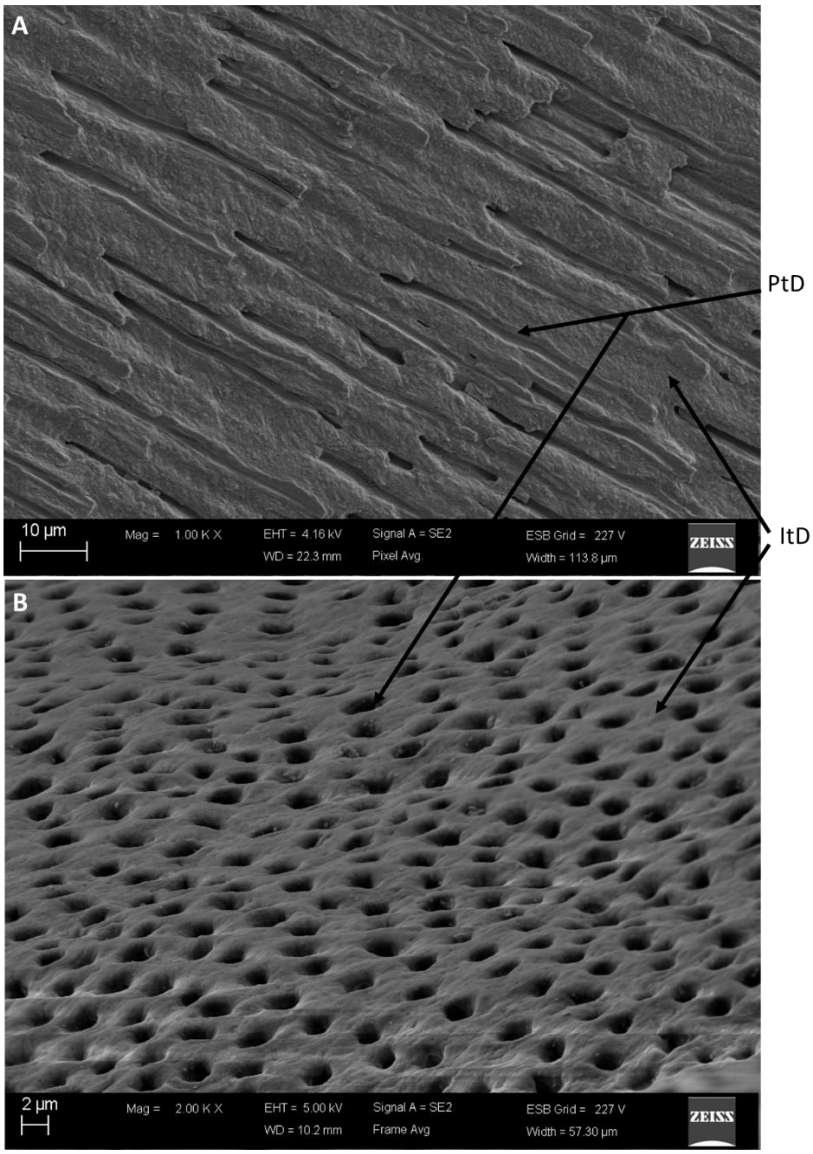

Figure 9 : **Human dentin by scanning electronic microscopy (SEM). A. cutting line is parallel to dentin tubules, B. cutting line is perpendicular to dentin tubules. PtD: peritubular dentin, ItD: intertubular dentin.** (Own data)

1.2.3.2 Dentin proteins

1.2.3.2.1 Collagens

In the dentin ECM, collagens form a 3D scaffold which is very important in dentinogenesis. Type I collagen is the major type in dentin matrix collagens (90%), other types of collagen were identified but at lower levels (1-3%) like types III and V collagens (Michel Goldberg and Smith 2004; Vital et al. 2012).

Collagen I formed by gathering of two α1 (I) chains and one α2 (I) chain. These chains entwine to form a triple helix of coiled coil framework (Rest and Garrone 1991). The

odontoblasts secrete thin collagen fibril subunits at their apical pole. Lateral fibril subunits assembly leads to fibrillar growth and then straight integration leads to the collagen lengthening**.**

1.2.3.2.2 Noncollagenous proteins (NCPs)

Noncollagenous proteins (NCPs) constitute the remaining 10% of the ECM scaffold and play an essential role in the regulation of bone and dentin mineralization. NCPs are divided into phosphorylated and nonphosphorylated NCPs.

1.2.3.2.2.1 Phosphorylated NCPs

SIBLINGs (Small Integrin Binding LIgand N-linked Glycoproteins), are a phosphoprotein family in which mutations are associated with abnormal phenotypes in the mineralization of bone and/or dentin (Qin, Baba, and Butler 2004; Vital et al. 2012). This family includes dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1), bone sialoprotein (BSP), matrix extracellular phosphorylated glycoprotein (MEPE), and osteopontin (OPN). All SIBLINGs were identified in dentin and bone ECM, but a high rate of DSPP expression was shown to be specific to dentin. The SIBLING members carry an arginine– glycine–aspartate cell adhesion domain (RGD) and a highly conserved acidic serine and aspartate-rich motif (ASARM) (P. S. Rowe et al. 2000; Fisher and Fedarko 2003). Noteworthy, the function of ASARM domain in bone and teeth mineralization (apatite crystals nucleator or inhibitor) is at present debated by the scientific community, in particular its implication in pathological processes such as inherited rickets (Addison and McKee 2010; David and Quarles 2010; P. S. N. Rowe 2012). It is of interest that, in addition to binding integrins SIBLINGs, may also specifically bind and activate several MMPs in the ECM suggesting that they could be involved in dentin matrix degradation (Fedarko et al. 2004).

1.2.3.2.2.2 Nonphosphorylated NCPs

The second group of NCPs is nonphosphorylated proteins, such as osteonectin (SPARC protein or BM40) and proteins with gamma-carboxylated glutamates (Gla) residues (osteocalcin and matrix Gla protein-MGP-). While osteonectin may contribute to the mineralization process, osteocalcin and MGP have been suggested to regulate HAP crystal nucleation (Bronckers et al. 1998; Onishi et al. 2005; Kaipatur, Murshed, and McKee 2008). The small leucine-rich proteoglycans (SLRPs), such as decorin, biglycan, fibromodulin, lumican, and osteoadherin, have also been identified in predentin and dentin (M. Goldberg, Septier, and Escaig-Haye 1987; M. Goldberg et al. 2003). They are thought to be involved in the transport of collagen fibrils through the predentin and in collagen fibrillogenesis (M.

Goldberg et al. 2003). Predentin is also rich in dermatan and chondroitin sulphate-containing (PG). It is of interest that adjacent to the mineralization front, predentin contains a large quantity of keratan sulphate-containing PG associated with a dramatic decrease in dermatan and chondroitin sulphate-containing PG. This switch in the proteoglycan type was attributed to MMP-3, which is closely related to a control of the dentin mineralization process (Hall et al. 1999).

1.2.3.3 Dentinogenesis

At the early stage of tooth development, the dental mesenchyme originates from the neural crest-derived mesenchyme migrate to the oral cavity under the oral epithelium and contribute to the tooth bud formation. During the last mitosis of the proliferate mesenchymal cells the cell located in contact with the basement membrane become preodontoblasts, whereas the daughter cells away from the basement membrane form the Hoehl's layer which constitutes a reservoir for replacing the damaged odontoblasts. After the differentiation odontoblasts become polarized and start to secret the extra cellular matrix components which will be the scaffold for hydroxyapatite (HAP) crystals deposition to form at the end the dentin.

Another classification showed that there are four dentins: Primary dentin, which is formed by odontoblasts which secret this dentin until the tooth becomes functional. Secondary dentin, is secreted by odontoblasts immediately after the end of primary dentin secretion (when the contact of antagonistic cusps is established), and continues throughout life. The major difference between primary and secondary dentins is morphological; in the secondary dentin the S-curve of the tubules is more accentuated. Tertiary reactionary dentin, is synthesized by odontoblasts or, if these cells are destroyed, by the subjacent cells of the Höehl‘s layer, as a reaction to carious decay, to abrasion or as a response to the release of some components of dental material fillings. Depending the severity and speed of the carious lesion, the age of the patient and the progression of the reaction, it appears as a layer of the osteodentin type, or as a tubular or atubular orthodentin. Tertiary reparative dentin is formed by pulp progenitors, implicated in the formation of a bone-like or in structure-less mineralization (pulp diffuse mineralization or pulp stones). These structures are closer to bone (osteodentin) rather than to dentin (Michel Goldberg et al. 2011).

1.2.4 Enamel

Enamel is the hardest and outer layer of tooth crown that protects the mammalian tooth from external chemical and physical effects. Enamel properties are associated with its special structural organization and connection with underlying dentin.(Janet Moradian-Oldak 2012).

Mature enamel consists of approximately 4% water and organic material and 96% inorganic materials (**Table 1**). Enamel inorganic content is a crystalline calcium phosphate (hydroxyapatite) which also is found in dentin, cementum, bone, and calcified cartilage (Antonio Nanci and Cate 2013).

Table 1 : **Percentage Wet Weight Composition of Rat Incisor Enamel. From** *(Antonio Nanci and Cate 2013)*

COMPONENT	SECRETORY STAGE (%)	MIDMATURATION (%)	LATE MATURATION (%)
Water	5	3	1
Mineral	29	93	95
Protein	66	4	4

The principal structural units of enamel are the rods (prisms) and interrod enamel (interprismatic substance) (**Figure 10**).

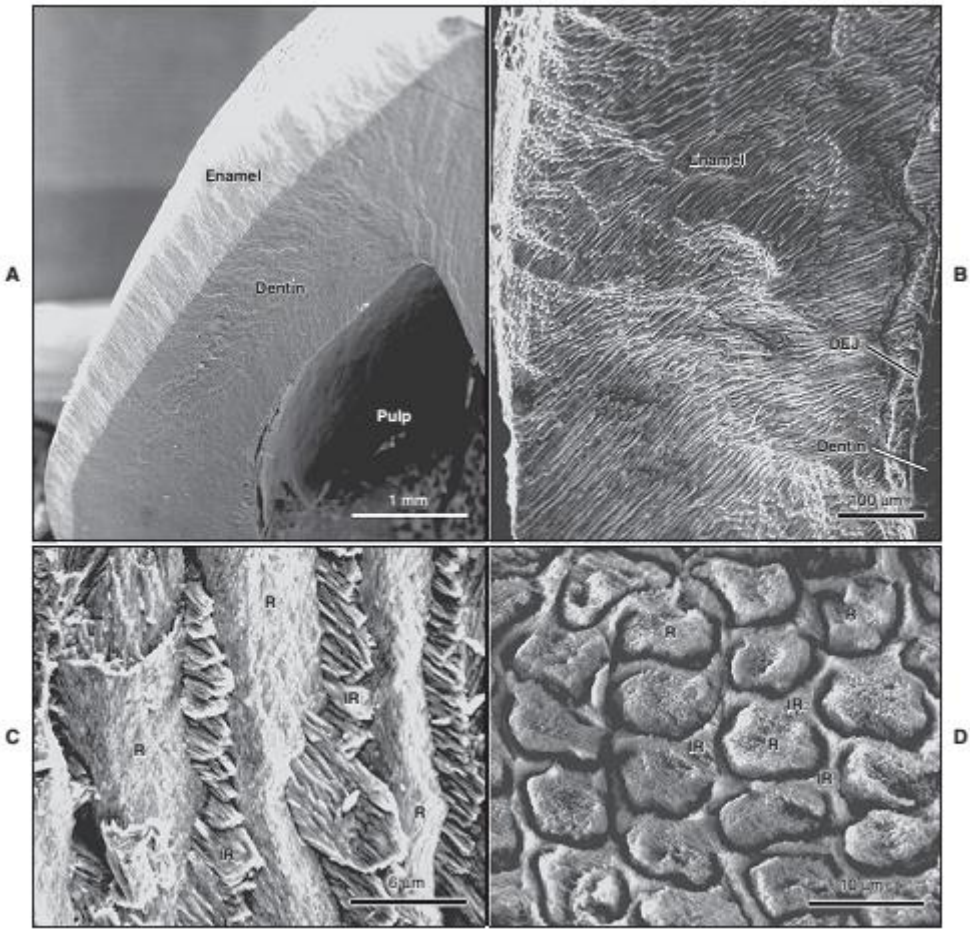

Figure 10 : **Scanning electron microscope views of (A) the enamel layer covering coronal dentin, (B) the complex distribution of enamel rods across the layer, (C and D)**

and perspectives of the rod-interrod relationship when rods are exposed longitudinally (C) or in cross section (D). Interrod enamel surrounds each rod. DEJ: Dentinoenamel junction; IR: interrod; R,rod. Adapted from (Antonio Nanci and Cate 2013)

1.2.4.1 Enamel proteins

Enamel proteins are synthesized by ameloblasts. During tooth development, the ameloblasts control the synthesis and secretion of the organic extracellular matrix (ECM) and then the biomineralization of this ECM. Enamel proteins are hydrophobic proteins known such as amelogenins and nonamelogenin proteins including ameloblastin, enamelin, tuftelin, tuft proteins, sulfated proteins and enamel proteases such as enamelysin (MMP-20) and KLK-4.

1.2.4.1.1 Amelogenin

Amelogenin gene exists only on the X chromosome in rodents (Snead et al. 1983; Chapman et al. 1991), while it exists on both X and Y chromosomes In human and cow (Lau et al. 1989).

Amelogenin constitutes more than 90% of the enamel protein content. It is secreted as a variety of isoforms because of alternative splicing of the amelogenin gene and processing of the parent molecules (C. W. Gibson et al. 1991; Lau et al. 1992), the major isoform is about 25 kDa. Amelogenin has a bipolar nature: it contains highly hydrophobic domains and hydrophilic N- and C-terminal sequences and this bipolar nature allows amelogenin monomers by self-assembly to form supramolecular resulting in the formation of nanospheres which regulate crystal spacing (Fincham et al. 1994; Fincham and Simmer 1997). The N-terminal A-domain is involved in the formation of nanospheres, whereas the carboxy-terminal B-domain prevents their fusion to larger assemblies (J. Moradian-Oldak et al. 2000).

Amelogenin has signaling activities (Carolyn W. Gibson 2008; Veis 2003), especially the small isoform; leucine-rich amelogenin peptide (LRAP) (Warotayanont et al. 2008). Because of its potential to promote cell differentiation and its interaction with bone cells, it has been used in periodontal regenerative therapies.

Amelogenin is not essential for the initiation of mineralization, but is essential for the elongation of enamel crystals and the achievement of proper enamel formation, because in spite of its absence in KO mice, a thin layer of mineralized enamel is formed.

1.2.4.1.2 Ameloblastin

Ameloblastin constitutes about 5% of enamel protein. Its expression significantly decrees during enamel maturation. The isolation of this protein is so difficult for several reasons, the

limitations in expression and hydrolysis by enamel proteinase MMP-20 as soon as secreted (Iwata et al. 2007; Yasuo Yamakoshi, Hu, Zhang, et al. 2006). In the ameloblastin KO mice, ameloblasts detach from the surface of the developing teeth, suggesting a potential role for ameloblastin in ameloblasts adhesion to the forming enamel (Fukumoto et al. 2004).

1.2.4.1.3 Enamelin

Enamelin is the largest enamel protein and constitutes about (3–5%) of enamel proteins. It is a phosphorylated, glycosylated protein and is rapidly cleaved following its secretion. The intact protein is only observed at the mineralization front, so it proposed to be implicated in crystal elongation (C. C. Hu et al. 1997; C. C. Hu et al. 2000).

Enam gene mutations cause an autosomal dominant forms of *amelogenesis imperfecta* AI (Hart et al. 2003) and no true enamel layer is formed in the Enam KO mice(J. C.-C. Hu et al. 2008).

Recently, it was reported that a large increase or decrease in enamelin expression impairs the production of enamel crystals and the prism structure (J. C.-C. Hu et al. 2014).

Enamelin and ameloblastin appear to have similar roles like crystallite initiation and elongation, whereas amelogenin appears to form a framework to allow the continued elongation of the already initiated crystallites (John D. Bartlett 2013).

1.2.4.1.4 Tuftelin

Tuftelin is expressed early at the bud stage of tooth development (several days befor the onset of mineralisation) so it is suggested to play a nucleator role during crystals formation. Its expression is also detected in several organs kidney, lung, liver, and testis (Zeichner-David et al. 1997; MacDougall et al. 1998).

1.2.4.1.5 Sulfated enamel proteins

Sulfated enamel proteins constitute an acidic nature family of proteins with unknown roles. They are difficult to be detected because of their presence in a small amount (C. E. Smith et al. 1995).

1.2.4.1.6 Amelotin

Amelotin is a glycoprotein recently discovered, its role is not yet clear (Iwasaki et al. 2005). It is expressed during the secretory stage of enamel development (Gao et al. 2010). Alternatively spliced variants lead to several isoform of amelotin.

1.2.4.1.7 Biglycan

Biglycan expression is in the dentin and the enamel (Septier et al. 2001). It is expressed by ameloblasts during tooth development, (M. Goldberg, Septier, Rapoport, et al. 2002), where it acts as an amelogenin expression repressor (M. Goldberg, Septier, Rapoport, et al. 2002; M. Goldberg et al. 2005b).

1.2.4.2 Enamel proteinases

Enamel proteinases are so important for the digestion of enamel proteins and enamel maturation. It was found that some of these proteinases have an ameloblast differentiation-dependent expression (Lu et al. 2008).

1.2.4.2.1 Matrix metalloproteinase 20 (MMP-20)

Enamelysin (MMP-20) is expressed by ameloblasts and odontoblasts (J. D. Bartlett et al. 1996; Fukae and Tanabe 1998), it is expressed from the beginning of secretion stage through the beginning of maturation stage of enamel and cleaves amelogenin, enamelin, and ameloblastin into stable intermediate products (Lacruz et al. 2011). In vitro studies showed that Mmp-20 stimulates the formation of nanorod structures formed by co-assembly of the parent amelogenin with its proteolytic products (X. Yang et al. 2011). Such assembly alteration was proposed to be related with the elongated growth of apatite crystals. It has been proposed that Mmp-20 activity produces protein intermediate products that will stimulate phase transformation of amorphous calcium phosphate nanoparticles into mineralized hydroxyapatite (Kwak et al. 2009).

1.2.4.2.2 Kallikrein-4 (KLK4)

Klk-4 is expressed from the end of secretory stage and throughout the maturation stage of enamel (Lacruz et al. 2011). Its function is to digest the intermediate products of amelogenin, enamelin and ameloblastin resulting from the MMP-20 action and facilitates enamel proteins removal which is necessary for enamel maturation and hardening (O. Ryu et al. 2002).

KLK-4 digests the 32-kDa enamelin fragment which is resistant to Mmp-20 action, (Yasuo Yamakoshi, Hu, Fukae, et al. 2006) and its activity is not affected like MMP-20 by the presence of apatite crystals in vitro (Z. Sun et al. 2010).

1.2.4.2.3 Other proteinases

1.2.4.2.3.1 Caldecrin (Ctrc)

Caldecrin Ctrc expression pattern in enamel is similar to Klk4, but lower, and it is predominantly expressed in the maturation stage of amelogenesis (Lacruz et al. 2011).

1.2.4.2.3.2 MMP-2

It has been demonstrated that recombinant MMP-2 cleave amelogenin into several fragments in vitro (Caron et al. 2001). MMP-2 also degraded most forms of amelogenin, suggesting that MMP-2 can participate, with MMP-20, to achieve complete amelogenin processing (Bourd-Boittin et al. 2005).

1.2.4.2.3.3 MMP-9

Recently, it was proposed that MMP-9 involved in enamel formation and controlling the processing of amelogenin (Feng et al. 2012)

1.2.4.3 Enamel formation

1.2.4.3.1 Pre-secretory stage

At this stage, ameloblasts start expressing very small amounts of enamel proteins even before the basement membrane break up and send cytoplasmic projections through the gaps directly after basement membrane disintegrate. With the disappearance of the basement membrane, dentin starts to mineralize and the apical surfaces of ameloblasts connect with the superficial collagen fibrils of the mantle dentin (Meckel, Griebstein, and Neal 1965; Cevc et al. 1980) (**Figure 12**).

1.2.4.3.2 Secretory Stage

At the beginning, ameloblasts secrete enamel proteins on top of and around existing dentin crystals initially and then around enamel crystals and into the space of disappeared basement membrane (**Figure 11**.A). With the continued secretion of enamel matrix, ameloblasts move back to create the necessary space for continuous deposition of enamel end this moment we can distinguish the initial enamel layer which is aprismatic (not separated into rod and interrod enamel)

Secretory ameloblasts develop a novel cell extension called Tomes' process at their apical (secretory) ends. This extension which has secretory and nonsecretory regions provides the architectural basis for organizing enamel crystals into rod and interrod enamel, (Meckel, Griebstein, and Neal 1965; Cevc et al. 1980).

Secretory ameloblasts secrete enamel proteins which concentrate along the ameloblast secretory membrane and form a mineralization front (there is no pre-enamel like the predentin in dentin or osteoid in bone). The mineralization front retreats with the Tomes' process as the enamel crystals grow in length (4µm/day) (Risnes 1986), and the ameloblasts

continue their secretion of enamel proteins (**Figure 12**). During this stage enamel crystals grow primarily in length and the enamel layer thickens.

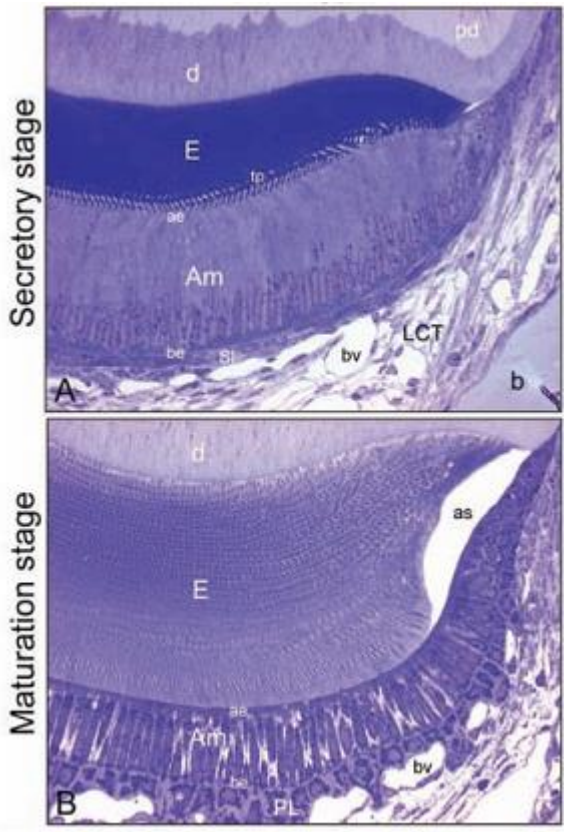

Figure 11 : **Semi-thin (0.5 µm) sections from glutaraldehyde-fixed, decalcified, and plastic embedded mandibular incisors of wild-type mice stained with toluidine blue to illustrate the appearance of enamel and enamel organ cells at mid-secretory stage (A) and near-mid-maturation stage (B) of enamel development. Abbreviations: E, enamel; Am, ameloblast; Si, *stratum intermedium*; pd, predentin; D, dentin; ae, apical end; be, basal end; bv, blood vessel; as, artifact space; b, bone; c, cementum. Adapted from** *(J. D. Bartlett and Smith 2013)*

1.2.4.3.3 Maturation Stage

At the end of secretory stage, enamel layer has its final thickness and ameloblasts reduce their secretion of enamel proteins (**Figure 11**.B), and start the secretion of KLK-4 which finishes the degradation of the organic matrix. The degradation and removal of growth-inhibiting enamel proteins terminate the growth of enamel crystallites in length, and accelerate their growth in width and thickness by the ion deposition on the thin crystals sides until they press against one another (C. E. Smith 1998). This process is necessary to have a harde and mature

enamel layer, and is directed by modulating ameloblasts that cycle through smooth and ruffle-ended phases.

During maturation stage a basal lamina is secreted at the base of the ameloblasts (**Figure 12**). Recently amelotin (AMTN) has been identified as one of the components of this basal lamina (Iwasaki et al. 2005; Moffatt et al. 2006).

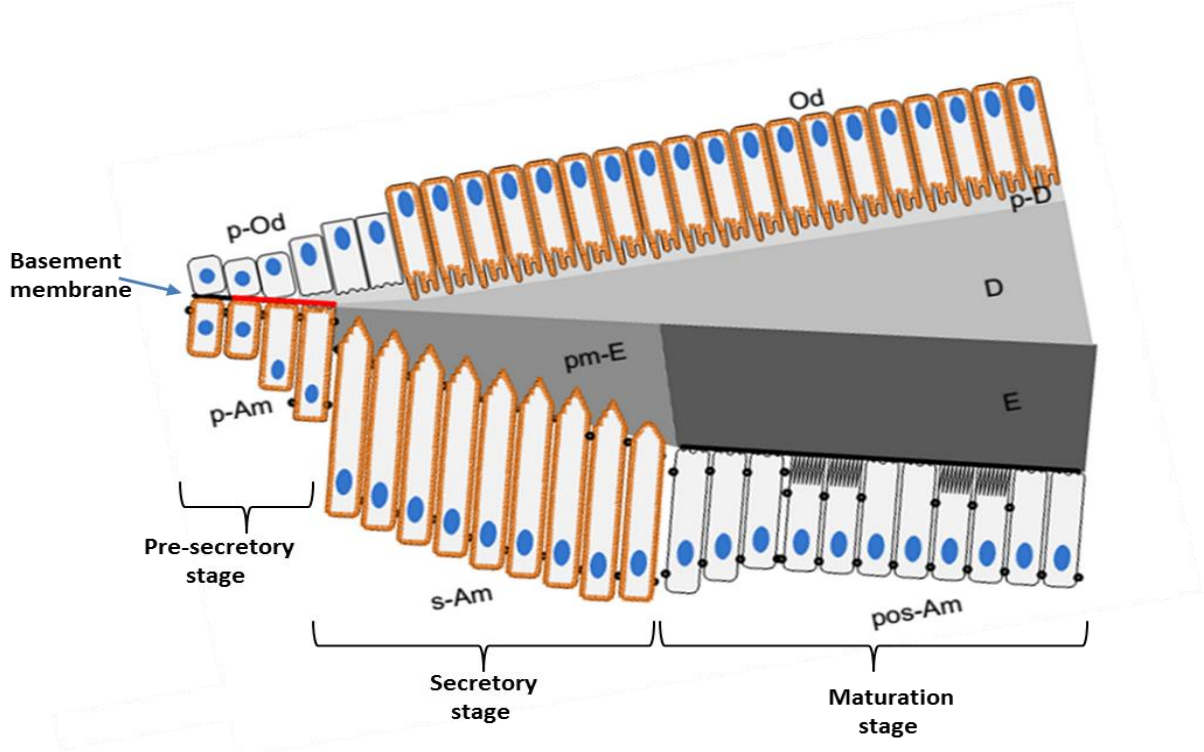

Figure 12 : **Schema present incisor enamel and denin formation. p-Am: pre-ameloblast; pOd: pre-odontoblast; s-Am: secreting Ameloblasts; od: odontoblasts; pos-Am: post-secretory ameloblasts; pD: predentin; D: dentin; pm-E: premature enamel; E: enamel. Adapted from** *(Khaddam et al. 2014)*

1.3 Matrix MetalloProteinases MMPs

MMPs is subdivided into soluble and membrane-type MMPs (MT-MMPs). The soluble MMPs are expressed as pro-enzymes that will be activated in the extracellular environment. MT-MMPs are intracellularly activated and identified as activators of soluble MMPs and were shown to be able to degrade extracellular matrix proteins ECM (Hamacher, Matern, and Roeb 2004).

In addition to the inhibition by endogenous inhibitors (tissue inhibitor of MMPs TIMP) or to the proteolytic activation of pro-MMPs, MMPs are regulated by cytokines or growth factors transcriptionally (Tsuruda, Costello-Boerrigter, and Burnett 2004)

MMPs are implicated in inflammation by regulating the availability and the activity of cytokines, chemokines, and growth factors, as well as integrity of tissue barriers. MMPs are also involved in tumors (Nissinen and Kähäri 2014).

1.3.1 MMPs and teeth

1.3.1.1 In physiological processes (development)

Several MMPs have been detected in developing tooth tissues (Michel Goldberg et al. 2003). They play a central role in the disruption of basement membrane. MMPs are also implicated in the functional regulation of growth factors and their receptors, cytokines and chemokines, adhesion receptors and cell surface proteoglycans, and a variety of enzymes (H. Li et al. 2002). MMPs participate in the remodeling of the ECM during tooth development to facilitate the migration of cells and the mesenchymal condensation (Chin and Werb 1997) and participate in the regulation of the mineralization process of dental hard tissues by cleaving the matrix proteins of the dentin and the enamel matrix (Simmer and Hu 2002; Fanchon et al. 2004).

MMP-1, -2, -3, -9 and MT1-MMP have been detected during tooth development, indicating that these MMPs have roles in tooth morphogenesis and eruption (Chin and Werb 1997; Sahlberg et al. 1992b; Caron, Xue, and Bartlett 1998; Randall and Hall 2002; Yoshiba et al. 2003).

1.3.1.2 In pathological processes

1.3.1.2.1 Periodontitis

High MMPs levels were detected in the periodontitis and apical periodontitis leading to accelerated matrix degradation, (de Paula e Silva et al. 2009; Paula-Silva, da Silva, and Kapila 2010). Collagenases (MMP-1, MMP-8, and MMP-13) and gelatinases (MMP-2 and MMP-9) are implicated in the digestion of collagen in the bone and periodontal ligament (Andonovska, Dimova, and Panov 2008; Corotti et al. 2009).

1.3.1.2.2 In the caries process

We developed this point (**Figure 13**) in Chaussain, Boukpessi**, Khaddam** et al, 2013 (end of introduction).

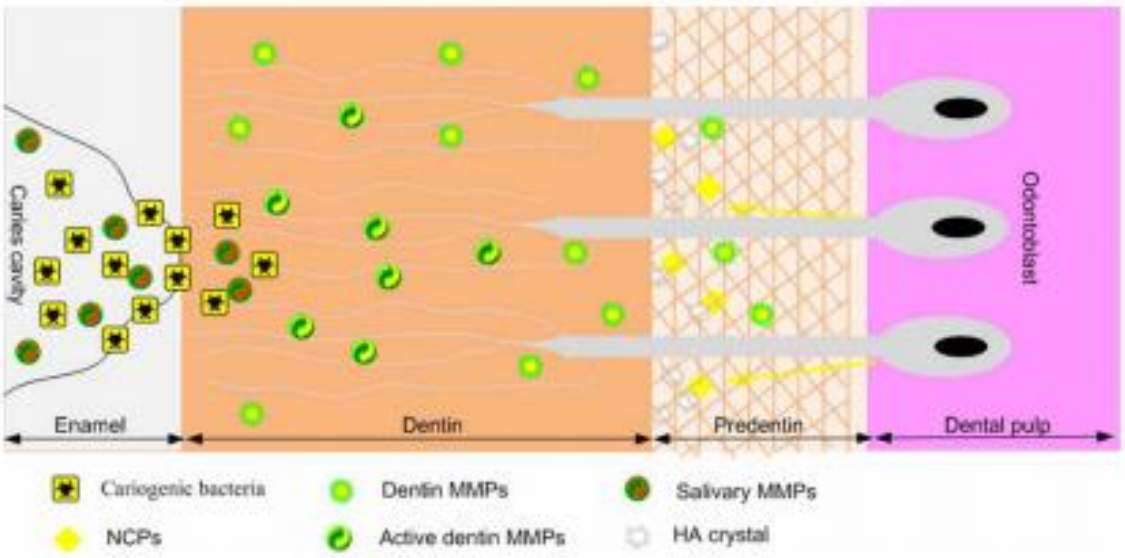

Figure 13 : **Schematic representation of MMP activity during the dentin carious process. Cariogenic bacteria present in the caries cavity release acids such as lactic acid that reduce the local pH. The resulting acidic environment demineralizes the dentin matrix and induces the activation of host MMPs derived from dentin or saliva (which bathes the caries cavity). Once the local pH is neutralized by salivary buffer systems, activated MMPs degrade the demineralized dentin matrix. Adapted from** *(Chaussain et al. 2013)*

1.4 EMMPRIN (Basigin,CD147)

1.4.1 Historic

Extra cellular matrix metalloproteinase inducer EMMPRIN (CD147), a member of the immunoglobulin superfamily, was described for the first time on the surface of solid tumor cells as an inducer of a various (MMPs in adjacent fibroblasts (Biswas 1982). Based on these latter properties it was named extracellular matrix metalloproteinase inducer EMMPRIN (Biswas et al. 1995). Previously EMMPRIN had different names including tumor cell-derived collagenase stimulatory factor (TCSF), Basigin, CD147, gp42, HT7, neurothelin, 5A11, OX-47 and M6 (T. Muramatsu and Miyauchi 2003).

1.4.2 Structure

1.4.2.1 Transmembrane form

EMMPRIN (Basigin) has four isoforms (basigin-1 to -4), caused by alternative transcription initiation and variation in splicing (**Figure 14**)(Belton et al. 2008) and the major isoform is basigin-2. EMMPRIN is highly glycosylated, Its protein portion is 27 kDa, and its

glycosylated form is 43 to 66 kDa (Miyauchi et al. 1990) and the nonglycosylated form has the ability to induce MMP expression in fibroblasts as the glycosylated form(Belton et al. 2008)

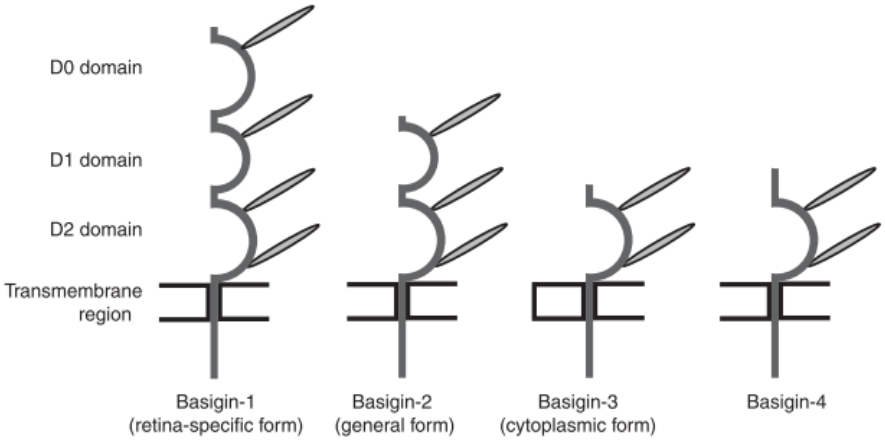

***Figure 14**:* **Basigin isoforms. Characteristic features of isoforms are mentioned within blanket. Carbohydrates are shown by light grey color. Adapted from** *(Takashi Muramatsu 2012)*

EMMPRIN is largely composed of three domains, extracellular immunoglobulin domain, a transmembrane domain and a cytoplasmic domain.

The extracellular domain has two immunoglobulin domains (a N-terminally located D1 domain and a more C-terminally located D2 domain) (**Figure 15**) and three potential Asparagine (Asn)-glycosylation sites; one in D1 domain and two in D2 domain (Miyauchi et al. 1990; Miyauchi, Masuzawa, and Muramatsu 1991).

The transmembrane domain has glutamic acid in its center, and is completely conserved between human, mouse and chicken (Miyauchi, Masuzawa, and Muramatsu 1991), this domain is important for interactions with other proteins in the same membrane.

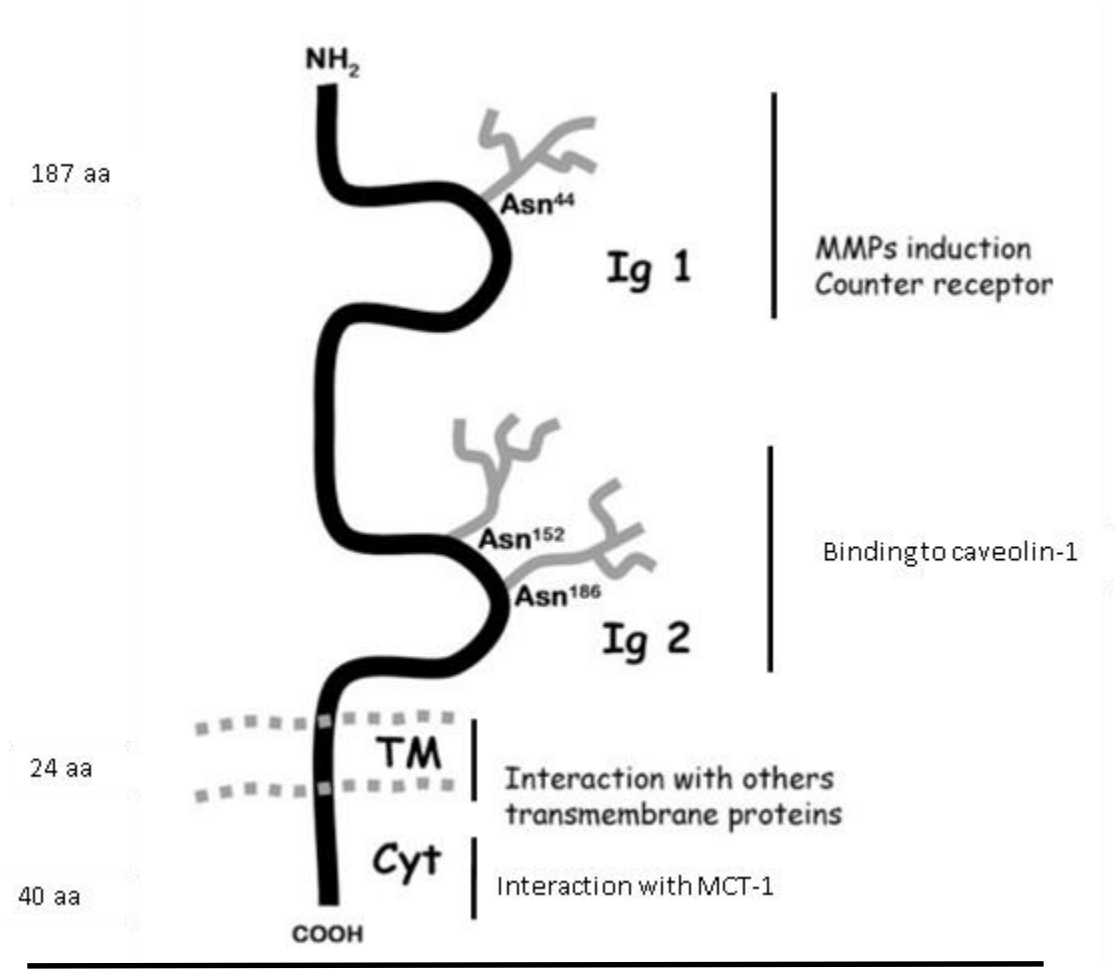

Figure 15: **Scheme of EMMPRIN structure. EMMPRIN contains an extracellular domain composed of two Ig loops with three Asn-linked oligosaccharides and short single transmembrane domain (TM) and a cytoplasmic domain (Cyt). The first Ig domain is required for counter-receptor activity, involved in MMP induction. Adapted from** (Gabison et al. 2009).

1.4.2.2 Soluble form

The soluble form of CD147 has been detected in conditioned media as:

- full-length protein (Taylor et al. 2002)
- or as part of shed microvesicles (Sidhu et al. 2004)
- as well as in forms lacking the transmembrane and cytoplasmic domain derived from MMP mediated cleavage of CD147 from the cell surface (Haug et al. 2004; Y. Tang et al. 2006; Egawa et al. 2006)

1.4.3 Phenotypes of EMMPRIN knock out (KO) mice

EMMPRIN KO mice have a low reproduction level which is at a much lower frequency than that expected by Mendelian segregation, KO embryos develop normally during blastocyst stage but at the time of implantation, about 75% of the null embryos are lost (Igakura et al. 1998) and half of the surviving mice had interstitial pneumonia and died within 4 weeks after birth (Igakura et al. 1998). EMMPRIN KO mice have a defect in the capability of implantation of the uterus (female), arrested spermatogenesis (male) (Igakura et al. 1998; Kuno et al. 1998), abnormal behavior (Naruhashi et al. 1997), deficits in vision (Hori et al. 2000) and a decreased response to odor (Igakura et al. 1996).

1.4.4 EMMPRIN interactions

Three possible EMMPRIN interactions were descriped (**Figure 16**):

- Homophilic cis interaction between EMMPRIN molecules within the plasma membrane of the same tumor cell (Yoshida et al. 2000).
- Homophilic trans interaction between EMMPRIN molecules on tumoral cells.(J. Sun and Hemler 2001)
- Heterophilic interactions between EMMPRIN molecule on a tumor cell and a putative EMMPRIN receptor on a fibroblast.

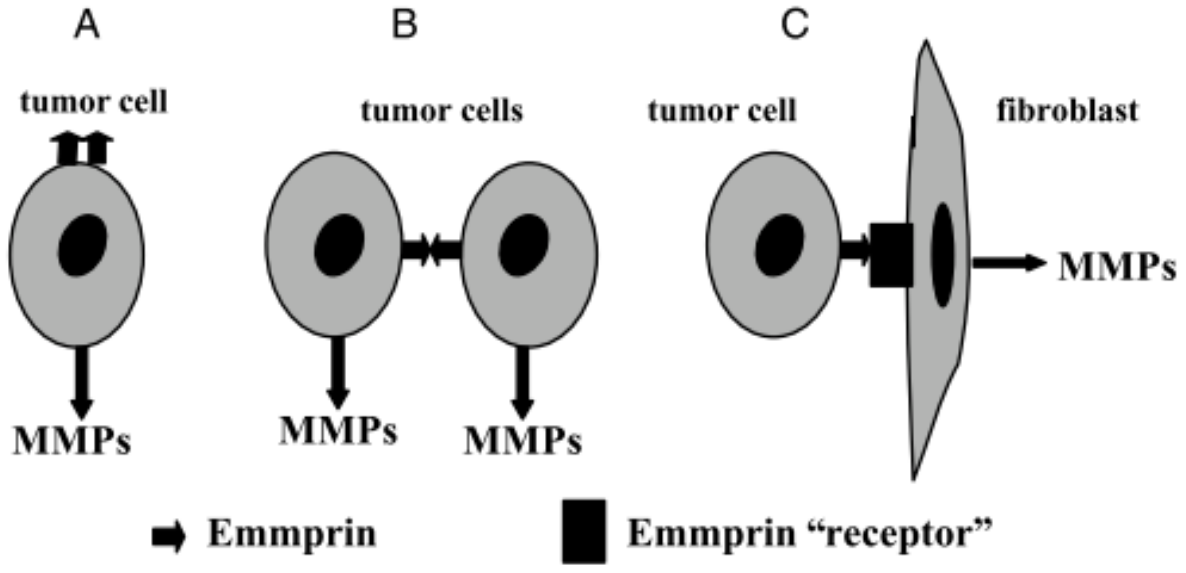

Figure 16: **Possible EMMPRIN-mediated interactions stimulating MMP production. (A) Homophilic cis interaction between EMMPRIN molecules within the plasma membrane of a tumor cell. (B) Homophilic trans interaction between EMMPRIN molecules on apposing tumor cells. (C) Heterophilic interactions between EMMPRIN**

on a tumor cell and a putative EMMPRIN receptor on a fibroblast. Adapted from (Toole 2003)

1.4.4.1 EMMPRIN Interactions with its binding partners within cell membrane

1.4.4.1.1 Monocarboxylic acid transporters (MCTs)

EMMPRIN has multiple binding partners, one of them, a family of monocarboxylic acid transporters (MCTs) (Kirk et al. 2000; Halestrap 2012) which transport monocarboxylic acids such as lactate, pyruvate and ketone bodies into and from the cells. Among the four MCTs (MCT1, MCT2, MCT3 and MCT4), EMMPIN binds to MCT1, MCT3 and MCT4 in the same membrane, and is essential for their transfer to the cell surface. An EMMPRIN dimer binds two MCT1 (Wilson, Meredith, and Halestrap 2002).

1.4.4.1.2 Integrins

It was shown that EMMPRIN associates with integrin α3 β1 and α6 β1 in the same membrane (Berditchevski et al. 1997), for example in extraembryonic membrane apposition in D. melanogaster (Reed et al. 2004) and in the visual system of D. melanogaster (Curtin, Meinertzhagen, and Wyman 2005).

1.4.4.1.3 Caveolin-1

Caveolin is a family of proteins form the major constituents of caveolae, within the plasma membranes of most cells that mediate the transcytosis of macromolecules in a clathrin-independent manner (Williams and Lisanti 2005) and comprised of three isoforms (caveolins 1, 2 and 3), only one of them caveolin-1 has been shown to associate with EMMPRIN. The association starts within the Golgi apparatus, where caveolin-1 binds to and guard the lower glycosylated forms of EMMPRIN to the plasma membrane, thus prevents the formation of, highly glycosylated species of EMMPRIN by the self-aggregating, which is responsible for MMP production (W. Tang, Chang, and Hemler 2004).

Caveolin-1 serves as a negative regulator of EMMPRIN; by the direct association with the second Ig domain of EMMPRIN which decrease EMMPRIN clustering and resulted in decreased MMP production (W. Tang and Hemler 2004).

Recently, an opposite effect of caveolin-1 is demonstrated. The increased caveolin-1 expression results in an increased proportion of highly glycosylated EMMPRIN relative to the lower glycosylated form and increased production of MMP-11 and higher invasiveness. In the same study down-regulation of caveolin-1 resulted in a decrease in highly glycosylated

EMMPRIN (Jia et al. 2006). Regardless of the outcome of these studies, the expression of EMMPRIN glycosylation forms is functionally linked with caveolin-1 expression.

1.4.4.2 EMMPRIN interactions with external molecules

1.4.4.2.1 Cyclophilin

Cyclophilin A is secreted from cells exhibit high level chemotactic activity to leukocytes and is involved in the inflammation, so it is the target protein of an immunosuppressive drug, cyclosporine A. Several studies confirmed that EMMPRIN is the receptor for cyclophilin A (V. Yurchenko et al. 2010; Vyacheslav Yurchenko et al. 2002), and for cyclophilin B (V. Yurchenko et al. 2010). The affinity between cyclophilin A and the extracellular region of EMMPRIN is weak, but it is strong with the transmembrane region (Schlegel et al. 2009).

1.4.4.2.2 EMMPRIN

Recently it has shown that nonglycosylated EMMPRIN ectodomains form dimer, and then interact with EMMPRIN on target cells (Belton et al. 2008). During internalization, EMMPRIN associates with another form of EMMPRIN (basigin-3 (Belton et al. 2008).

1.4.4.2.3 Platelet glycoprotein VI (GPVI)

Recently, platelet glycoprotein VI (GPVI) has been identified as a novel receptor for EMMPRIN and can mediate platelet rolling via (Seizer et al. 2009).

1.4.5 EMMPRIN functions

1.4.5.1 In physiological processes

1.4.5.1.1 Tissue repair/remodeling

The balance between MMP-induced stromal remodeling/restoration and stromal destruction is so delicate. EMMPRIN has been proposed as a mediator for this balance directly via a feedback mechanism that links the affected epithelial cells with neighboring fibroblasts (Gabison, Hoang-Xuan, et al. 2005).

In corneal ulcerations the protective epithelial barrier of the eye is damaged, leaving eye open to infection by bacteria, viruses and fungi and, if left untreated, they can result in blindness. EMMPRIN has been detected in both healthy and ulcerated corneas but was found at greater levels within ulcerated specially at the areas of greater MMP expression (Gabison, Mourah, et al. 2005).

Within the cardiovascular system, the balance is also delicate. MMP expression is critical for the prevention of hypertension and, at the same time, implicated in the progression of congestive heart failure (CHF) (Spinale et al. 2000; Ergul et al. 2004).

1.4.5.1.2 Chaperone functions

It was shown that In MCT-transfected cells, the MCT1 and MCT4 expressed protein accumulated in a perinuclear compartment, and it was found that co-transfection with CD147 enabled plasma membrane expression of active MCT1 or MCT4. Showing that EMMPRIN facilates proper expression of MCT1 and MCT4 at the cell surface and have a chaperone function (Kirk et al. 2000).

1.4.5.1.3 Implantation

Since MMPs are required in implantation (Alexander et al. 1996; Werb 1997), defective implantation result from mis-regulation of MMP production due to lack of EMMPRIN stimulation in EMMPRIN KO mice.

1.4.5.1.4 Spermatogenesis

EMMPRIN is strongly expressed in spermatocytes (Igakura et al. 1998) and its absence in EMMPRIN KO mice, leads to arrest spermatogenesis at the stage of differentiation of primary spermatocytes into secondary spermatocytes at the metaphase of the first meiosis (Igakura et al. 1998).

1.4.5.1.5 Retinal development and maintenance

EMMPRIN mediates MCTs transport to the plasma membrane so MCT1, MCT3 and MCT4 were found to be deficient at the plasma membrane of the retinal pigment epithelia, which leads abnormal photoreceptor function and blindness (Hori et al. 2000; Philp et al. 2003).

1.4.5.1.6 Cell interactions

EMMPRIN mediates the adhesive cell interactions, like in the embryonic retinal cell aggregation and influences glial cell maturation (Fadool and Linser 1993).

1.4.5.1.7 Hematopoetic cell activation and erythrocytes circulation

It was found that EMMPRIN plays a role in hematopoietic cell activation as during dendritic cell differentiation (Cho et al. 2001; Spring et al. 1997). Furthermore, It has been shown that EMMPRIN is expressed in erythrocyte lineage cells, including mature erythrocytes and blocking EMMPRIN by the injection of a monoclonal antibody in the mice causes selective trapping of erythrocytes in the spleen (Coste et al. 2001).

1.4.5.1.8 Other

EMMPRIN is implicated in other several physiological processes like neural network formation and development (Schlosshauer 1991; Fadool and Linser 1993), restriction of synaptic bouton size (Besse et al. 2007), calcium transport (J. L. Jiang et al. 2001), neutrophil chemotaxis (Vyacheslav Yurchenko et al. 2002), and blood –brain barrier development (Schlosshauer 1993).

1.4.5.2 In pathological processes

1.4.5.2.1 Cancer

High levels of EMMPRIN were reported in numerous malignant tumors including bladder, skin, lung and breast carcinoma, and lymphoma (Polette et al. 1997; Bordador et al. 2000; Thorns, Feller, and Merz 2002), and were also associated with poor prognosis (Kanekura, Chen, and Kanzaki 2002; Davidson, Givant-Horwitz, et al. 2003; Davidson, Goldberg, et al. 2003; Ishibashi et al. 2004).

EMMPRIN induces several malignant properties associated with cancer. These include:

1.4.5.2.1.1 MMPs

Tumorigenic cells expressing EMMPRIN induce MMP expression by neighboring stromal cells (**Figure 17**) (Biswas 1982; Kataoka et al. 1993; Biswas et al. 1995) and regulates MMP production at the transcription level by a mitogen activated protein kinase(MAPK) p38 pathway (Lim et al. 1998; Lai et al. 2003). Both recombinant EMMPRIN and tumoral EMMPRIN have been shown to induce the expression of collagenase I (MMP-1), gelatinase A (MMP-2), stromelysin-1 (MMP-3), and membrane type 1- and type 2-MMPs (MT1- and MT2-MMP) by fibroblasts (Cao, Xiang, and Li 2009; J. Sun and Hemler 2001; R. Li et al. 2001; Sameshima et al. 2000).

In situ hybridization analyses of both tumor and peri-tumoral fibroblasts in different organs like breast, colon, lung, skin and head/neck tumors showed that EMMPRIN expression is primarily tumor associated, while MMP expression is fibroblast associated (Pyke et al. 1992; Majmudar et al. 1994; Noël et al. 1994; Heppner et al. 1996; Polette et al. 1997).

EMMPRIN can induce EMMPRIN and MMP expression in far stromal cells by its soluble form which result from proteolytic cleavage of the carboxy terminus, and is thought to help metastasis to distant sites (Y. Tang et al. 2004).

Recently, another mechanism for MMP stimulation in distant cells was described. It was found that EMMPRIN expressed by malignant testicular cells by membrane vesicles (MVs) secreted from these cells, can exert its MMP inducing effect on distant cells within the tumor microenvironment to promote tumor invasion (Milia-Argeiti et al. 2014).

These properties make EMMPRIN a good target in cancer therapy. It has been shown that antibodies to EMMPRIN can decrease MMP expression leading to an inhibition of tumor cell invasion (Bordador et al. 2000; J. Sun and Hemler 2001; Kanekura, Chen, and Kanzaki 2002).

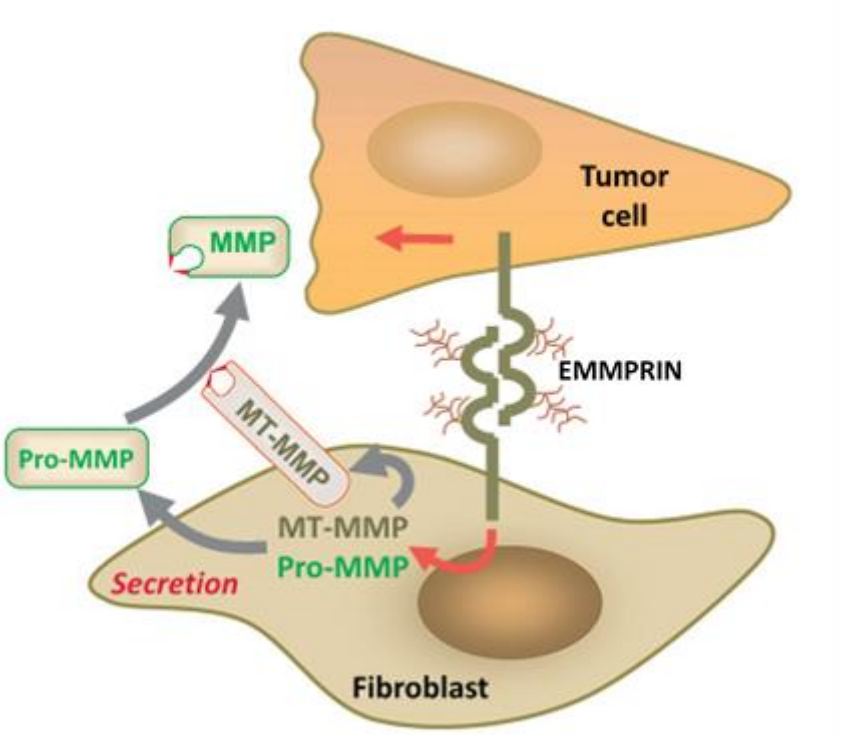

Figure 17 : **Tumor-cell induced activation of adjacent fibroblasts by homophilic EMMPRIN signaling. Adapted from** *(Joghetaei et al. 2013)*

1.4.5.2.1.2 VEGF

VEGF works as a major regulator of the angiogenic process in different circumstances, including tumor formation. EMMPRIN, in addition to increasing tumor invasion through MMP induction, it induces angiogenesis by the up-regulation VEGF expression (Y. Tang et al. 2005) as well as the stimulation of cell survival signaling, including Akt, Erk and FAK, through the increased production of the pericellular polysaccharide hyaluronan (Toole and

Slomiany 2008). EMMPRIN regulates VEGF production in tumor and fibroblast cells via the PI3K-Akt pathway (Y. Tang et al. 2006).

1.4.5.2.1.3 HIF-1α and MCT

The increase of tumor size makes the tumor microenvironment suffering from hypoxia and induce the hypoxia inducible factor, HIF-1 α , a transcription factor which has been shown to induce MCT-4 gene expression in cells (Moeller, Dumitrescu, and Refetoff 2005; Moeller et al. 2006). Up-regulation of MCTs in tumor cells is necessary for tumor survival and increase lactic acid concentration in the tumoral extracelluar microenvironment. This excess lactic acid inhibits peritumoral cytotoxic T cell function, and permitting continued uncontroled growth of the tumor (Fischer et al. 2007). MCT up-regulation is coordinated with EMMPRIN up-regulation which induces MMP production by peritumoral fibroblasts resulting in the extracellular matrix degradation and a favorable environment for tumor metastasis.

1.4.5.2.2 Rheumatoid arthritis

In rheumatoid arthritis (RA), Cyclophilin A (Cyp-A) up-regulates the expression of MMP-9 via the EMMPRIN signaling pathway through direct binding to EMMPRIN (Y. Yang et al. 2008). And recently, it was found that EMMPRIN induces up-regulation of HIF-1α and VEGF in RA fibroblast-like synoviocytes, which promotes angiogenesis, and leads to the persistence of synovitis (C. Wang et al. 2012).

1.4.5.2.3 Ischemic disease

The oxygen level decreases in the heart during myocardial infarction and in the brain during stroke. Because of hypoxia and ischemia cells become dependent upon glycolysis for energy metabolism, for continued cell viability the EMMPRIN associated lactate transporters MCT-1 and MCT-4 will be necessary (Kirk et al. 2000). High levels of MCT and EMMPRIN expression are detected under ischemic conditions in cardiac and neuronal cells (F. Zhang et al. 2005; Han et al. 2006), and it has been reported that EMMPRIN/Cyclofilin A association protects neurons from ischemia and hypoxia (Boulos et al. 2007).

1.4.5.2.4 Graft-versus-host disease

Using monoclonal antibody to EMMPRIN as a treatment for patients exhibiting acute graft-versus-host disease, shows promising efficacy, this effect due to suppression of leukocyte activation (Deeg et al. 2001)

1.4.5.2.5 Other diseases

Role of EMMPRIN is reported in other processes like atherosclerosis (L. Liang, Major, and Bocan 2002), heart failure (Y. Y. Li, McTiernan, and Feldman 2000; Spinale et al. 2000),

lung injury (Foda et al. 2001), viral infection (Pushkarsky et al. 2001), Alzheimer's disease (Zhou et al. 2005), chronic liver disease (Shackel et al. 2002) and in lymphocyte migration and activation (Koch et al. 1999; Renno et al. 2002; X. Zhang et al. 2002) .

1.4.6 EMMPRIN and tooth

1.4.6.1 In physiological processes

During tooth development, in cap stage, EMMPRIN expression was detected in the cell membranes of the inner enamel epithelium, *stratum intermedium* cells of the enamel organ and the dental papilla cells underlying the inner epithelium (**Figure 18**.a) (Kumamoto and Ooya 2006; Schwab et al. 2007; Xie et al. 2010; S.-Y. Yang et al. 2012). In bell stage, it was detected in ameloblasts, *stratum intermedium*, and in odontoblasts (**Figure 18**.b) (Schwab et al. 2007; Xie et al. 2010; S.-Y. Yang et al. 2012).

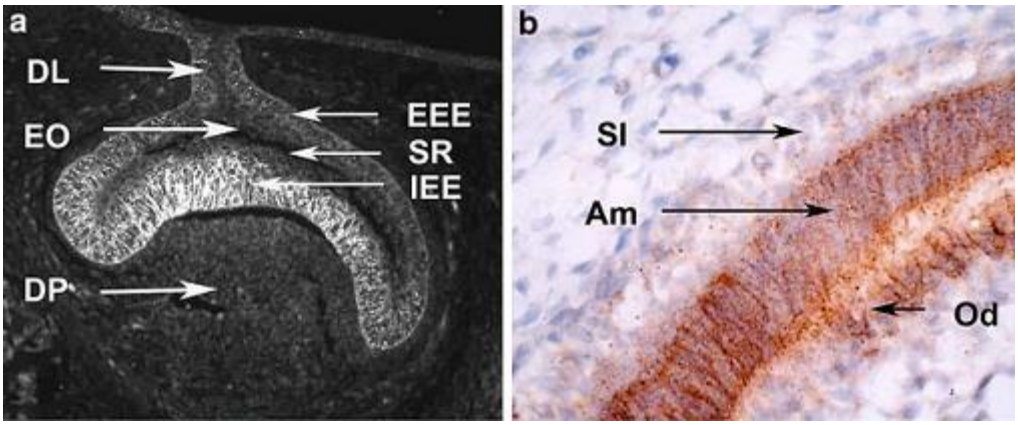

Figure 18 : **Immunoreactivity (IR) for EMMPRIN. a Cells of the inner enamel epithelium (cap stage of the enamel organ) show intense EMMPRIN IR (Alexa-coupled). b Ameloblasts as well as odontoblasts (bell stage of the enamel organ) exhibit strong EMMPRIN IR. Note the IR in the borderline between ameloblasts and the *stratum intermedium*. Mesenchymal cells of the dental papilla are only weakly immunoreactive. Abbreviations: A ameloblast; DL dental lamina; EEE external enamel epithelium; IEE internal enamel epithelium; EO enamel organ; Od odontoblast; SI *stratum intermedium*; SR *stellate reticulum*. Adapted from** *(Schwab et al. 2007)*

EMMPRIN variability during tooth development was investigated, and it was found that EMMPRIN mRNA expression was higher in E13.0 mouse mandible than that in E11.0 (**Figure 19**.a). and was higher in P1 mouse tooth germ than that in E14.0 (**Figure 19**.b) (Xie et al. 2010).

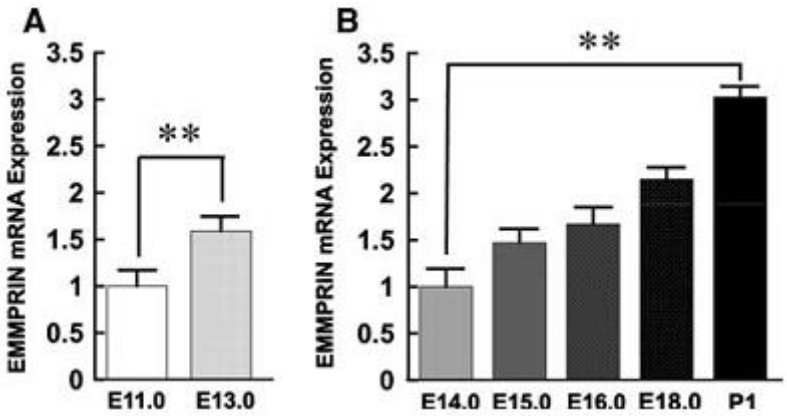

Figure 19 : **Transcription level of EMMPRIN in different stages of tooth development. a EMMPRIN mRNA was higher in E13.0 mandible than that in E11.0. b The expression of EMMPRIN mRNA was higher in P1 tooth germ than that in E14.0. Adapted from** *(Xie et al. 2010)*

At the root formation stage of tooth development, EMMPRIN was expressed strongly in the follicular cells overlaying the occlusal region of the rat molar germs. But, the expression was not region-specific and was weak in the follicular tissues in molar germs at the cap stage. So it was suggested that EMMPRIN play role in dental hard tissue maturation and the formation of an eruption pathway (S.-Y. Yang et al. 2012).

The differentiation-dependent co-expression of EMMPRIN with MMPs in the odontoblasts and enamel organ indicates that EMMPRIN plays role in proteolytic enzymes induction in the rat tooth germ (Schwab et al. 2007). And it was found that EMMPRIN colocalizes with caveolin-1 in cell membranes of ameloblasts and in inner enamel epithelial cells (Schwab et al. 2007).

EMMPRIN functional role in tooth germ development was investigated, by an EMMPRIN siRNA interference approach. Significant increase in MT1-MMP mRNA expression and a reduction in MMP-2, MMP-3, MMP-9, MMP-13 and MT2-MMP mRNA expression were observed in the mouse mandibles following EMMPRIN abrogation. These results indicate that EMMPRIN could be involved in the early stage of tooth germ development and morphogenesis (**Figure 20**), possibly by regulating the MMP expression (Xie et al. 2010).

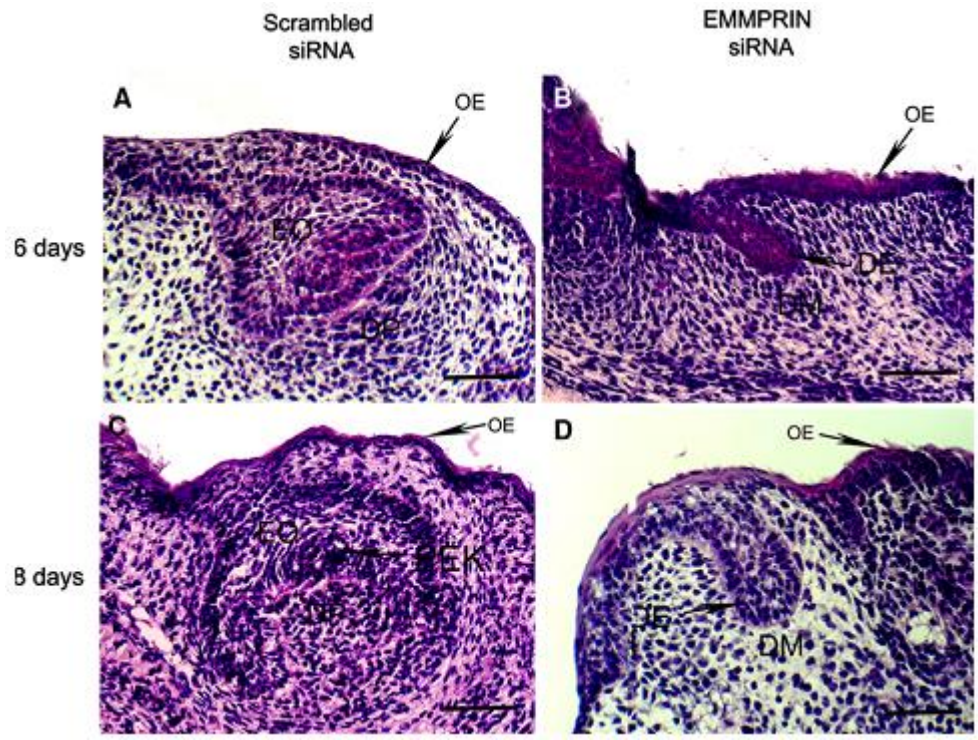

Figure 20 : **Examination the role of EMMRIN in early tooth germ development using EMMPRIN siRNA in the cultured mandible at E11.0. a After being cultured for 6 days, the tooth germ was found to have developed into the cap stage in mandibles cultured with scramble siRNA. b Dental epithelial bud was observed in the mandible treated with EMMPRIN siRNA after 6 days of culture. c A cap-like mature enamel organ was observed in the mandibles with scrambled siRNA supplement at 8-day culture. d EMMPRIN siRNAtreated mandible explants also showed a bud-like tooth germ at 8-day culture. EMMPRIN siRNA had a specific effect on the morphogenesis of tooth germ. DE dental epithelium, DM dental mesenchyme, DP dental papilla, EO enamel organ, OE oral epithelium, PEK primary enamel knot. Adapted from** *(Xie et al. 2010)*

1.4.6.2 In pathological processes

Several reports have pointed to the relation between periodontitis and EMMPRIN (Dong et al. 2009; Xiang et al. 2009; L. Liu et al. 2010; Feldman et al. 2011; D. Yang et al. 2013; J. Wang et al. 2014).

Elevated levels of EMMPRIN have been related to the progression of periodontal disease (Dong et al. 2009). EMMPRIN expression level increased from day 3 to day 7 and then gradually decreased from day 11 to day 21 (L. Liu et al. 2010; D. Yang et al. 2013).

During periodontitis development EMMPRIN was detected in the interdental gingiva, the gingival epithelium (Figure 21) and adjacent fibroblasts and in the inter-radicular bone. its

inflammation-dependent expression was associated with collagen breakdown and alveolar bone loss (L. Liu et al. 2010).

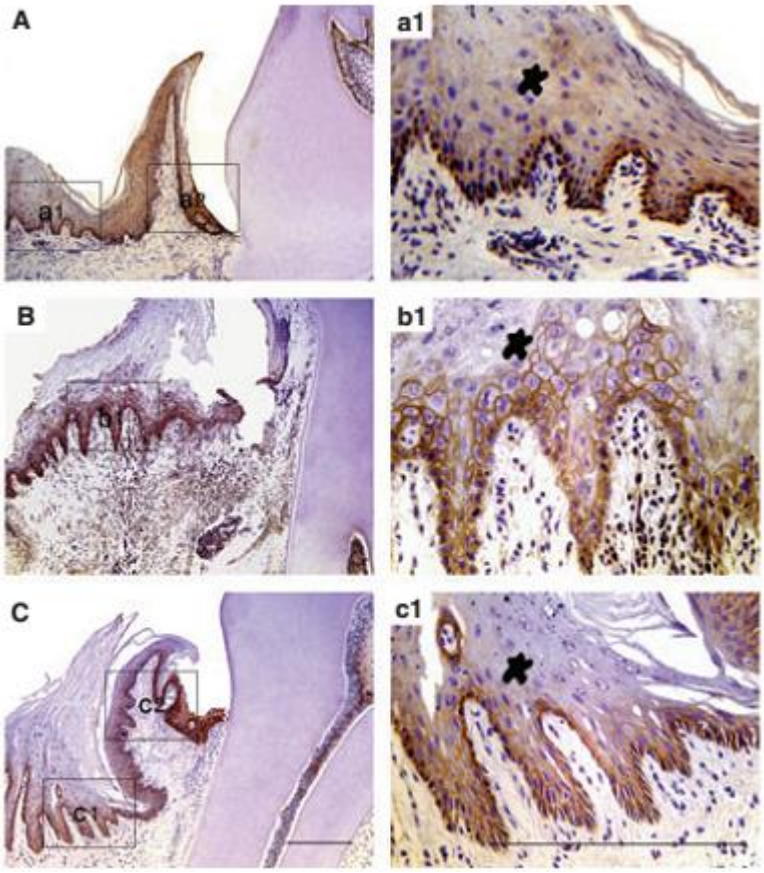

Figure 21 : **Temporal expression and localization of EMMPRIN in the gingival epithelium during ligature-induced periodontitis in the first mandibular molar of rats. (A) On day 0 (health), the immunoreactivity was strong in the basal cells, with a decrease toward the upper layers in the attached gingival epithelium (star in a1). (B) On day 7, immunoreactivity was greatly enhanced in the attached gingiva (star in b1). (C) On day 15, immunoreactivity was similar to that seen in the healthy state in the attached gingival epithelium (star in c1). Adapted from** *(L. Liu et al. 2010)*

EMMPRIN relation with other proteins during periodontitis was studied, it was found that EMMPRIN is associated with MMP-13 (higher expression level in day 3) but not with MMP-8 (higher MMP-8 expression in day 3) (D. Yang et al. 2013), and it was found that the increased active MMP-1 and proMMP-1 production in the chronic human periodontitis may be associated with elevated HG-EMMPRIN levels (J. Wang et al. 2014).

Soluble forms of EMMPRIN were shown to be present in gingival crevicular fluid (GCF) of patients with different periodontal diseases for the first time by Emingil et al. These authors showed that elevated EMMPRIN levels in gingival crevicular fluid were related to the

enhanced severity of periodontal inflammation, indicating that EMMPRIN may participate in the regulation of periodontal disease progression (Emingil et al. 2006).

High EMMPRIN level was detected in human ameloblastomas (L.-J. Jiang et al. 2008; Kumamoto and Ooya 2006; Er et al. 2001), oral squamous cell carcinoma (Bordador et al. 2000), and odontogenic cysts (L.-J. Jiang et al. 2008; Ali 2008). EMMPRIN expression was significantly higher in ameloblastomas than in odontogenic cysts, and microvessel density was positively associated with EMMPRIN expression to some extent (L.-J. Jiang et al. 2008). No significant difference in EMMPRIN expression was found among tumor types or subtypes (Kumamoto and Ooya 2006). EMMPRIN expression variability in various types of odontogenic cysts was studied and it was found that EMMPRIN expression was significantly higher in the epithelial lining of odontogenic keratocysts than in the dentigerous and periapical cysts (Ali 2008).

2 MMPs and dentin matrix degradation

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Dentin matrix degradation by host matrix metalloproteinases: inhibition and clinical perspectives toward regeneration

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Bacterial enzymes have long been considered solely accountable for the degradation of the dentin matrix during the carious process. However, the emerging literature suggests that host-derived enzymes, and in particular the matrix metalloproteinases (MMPs) contained in dentin and saliva can play a major role in this process by their ability to degrade the dentin matrix from within. These findings are important since they open new therapeutic options for caries prevention and treatment. The possibility of using MMP inhibitors to interfere with dentin caries progression is discussed. Furthermore, the potential release of bioactive peptides by the enzymatic cleavage of dentin matrix proteins by MMPs during the carious process is discussed. These peptides, once identified, may constitute promising therapeutical tools for tooth and bone regeneration.

Keywords: dentin, carious process, matrix degradation, MMPs, SIBLINGs, MMP inhibitors, biopeptides, regeneration

INTRODUCTION

Enamel is the hardest structure of the organism, containing about 96% of mineral but only traces of organic matrix (less than 1%). The enamel carious process involves physicochemical reactions, where the mineral is dissolved by acids liberated by cariogenic bacteria. In contrast, the dentin is less mineralized and contains an abundant organic material (19–20%), mainly composed of type I collagen. Therefore, the dentin carious process strongly differs from the enamel carious process. First, the bacterial acids dissolve the dentin mineral, which progressively uncovers the organic dentin extracellular matrix (ECM). Second, proteases degrade the ECM components allowing the progression of cariogenic bacteria toward pulp tissues. Such progression is facilitated by the tubular nature of the circumpulpal dentin. It has long been assumed that dentin organic matrix is degraded by proteases secreted by cariogenic bacteria. However, bacterial collagenases have been shown to be highly pH sensitive and not able to resist the acidic fall (pH 4.3) during the demineralization phase of a pH cycling model (Kawasaki and Featherstone, 1997), suggesting that this enzyme's contribution to dentin matrix degradation may be limited. Therefore, the potential role of host-derived proteases and in particular MMPs, in dentin matrix degradation has been introduced. MMPs form a mammalian family of ECM proteinases involved in normal and pathological events in almost all tissues of the organism including the tooth. MMPs present specific properties and characteristics (Brinckerhoff and Matrisian, 2002), including their ability to cleave matrix components, their dependence on a zinc ion for activity, the requirement that the enzymes be activated by the cleavage of a prodomain, the conservation of specific amino acid sequences between family members, and inhibition of their enzymatic activity by endogenous tissue inhibitors of metalloproteinases (TIMPs) (Visse and Nagase, 2003). Numerous studies have demonstrated endogenous proteolytic enzyme activity within the dentin, including matrix metalloproteinases MMP-2, MMP-9 and MMP-3 (Tjaderhane et al., 1998; Mazzoni et al., 2007) which were shown to be required for normal dentin formation. Once the neoformed dentin matrix mineralizes, some of these enzymes remain trapped in the calcified matrix, either under active or proenzyme forms (Palosaari et al., 2003). It can be hypothesized that the cariogenic demineralization process not only re-exposes these enzymes but also potentially induces their activation. In addition, the saliva, which bathes the dentin carious lesion, contains several proteases that can also participate in the organic matrix degradation (Tjaderhane et al., 1998; Van Strijp et al., 2003). The aim of the present article is to review the role of the host metalloproteinases (MMPs) in the dentin carious process. The ability of MMPs to liberate bioactive peptides and degrade dentin matrix proteins as well as the potential use of protease inhibitors to block their activity in the treatment of caries will be discussed.

DENTIN MATRIX PROTEINS

Dentin ECM consists of a 3D scaffold that is mainly formed of type I collagen fibrils (90%). Types III and V collagens were also identified in this scaffold but at lower levels (1–3%) (Goldberg

and Smith, 2004; Opsahl Vital et al., 2012). Thin collagen fibrils are secreted at the apical pole of the odontoblast body to form the predentin (which is equivalent to the osteoid matrix in bone). Collagen fibrils undergo fibrillogenesis along the predentin by processes of self-assembly and cross-linking to form a template which can be efficiently mineralized. Non-collagenous proteins (NCPs), which have been the focus of intense studies in the last decade because of their potential roles in the regulation of bone and dentin mineralization, constitute the remaining 10% of the ECM scaffold. It has been suggested that some NCPs are associated with specific sites on collagen fibrils to regulate the nucleation and growth of hydroxyapatite (HAP) crystals. For example, the glycoaminoglycans (GAG) part of the proteoglycans (PG) was shown to be important for the collagen fibril maturation by delaying apatite crystals deposition to allow the fibrils to reach a diameter sufficient to achieve proper mineralization (Embery et al., 2001). The importance of NCPs in the mineralization process has been well demonstrated by mutation studies and by experimental suppression of NCP genes. These experiments have highlighted the importance in the mineralization process of SIBLINGs (Small Integrin Binding LIgand N-linked Glycoproteins), a phosphoprotein family in which mutations are associated with abnormal phenotypes in the mineralization of bone and/or dentin (Qin et al., 2004; Opsahl Vital et al., 2012). This family includes dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1), bone sialoprotein (BSP), matrix extracellular phosphorylated glycoprotein (MEPE), and osteopontin (OPN). It appears that the associations of these highly phosphorylated and acidic NCPs with specific sites on collagen molecules are essential in promoting the nucleation and growth of apatite crystals. All SIBLINGs were identified in dentin and bone ECM, but a high rate of DSPP expression was shown to be specific to dentin. The SIBLING members carry an arginine–glycine–aspartate (RGD) cell adhesion domain and a highly conserved acidic serine and aspartate-rich motif (ASARM) (Rowe et al., 2000; Fisher and Fedarko, 2003). Noteworthy, the function of ASARM domain in bone and teeth mineralization (apatite crystals nucleator or inhibitor) is at present debated by the scientific community, in particular its implication in pathological processes such as inherited rickets (Addison and Mckee, 2010; David and Quarles, 2010; Rowe, 2012). It is of interest that, in addition to binding integrins SIBLINGs, may also specifically bind and activate several MMPs in the ECM suggesting that they could be involved in dentin matrix degradation (Fedarko et al., 2004).

Dentin contains non-phosphorylated proteins, such as osteonectin (SPARC protein or BM40) and proteins with gamma-carboxylated glutamates (Gla) residues (osteocalcin and matrix Gla protein-MGP-). While osteonectin may contribute to the mineralization process, osteocalcin and MGP have been suggested to regulate HAP crystal nucleation (Bronckers et al., 1998; Onishi et al., 2005; Kaipatur et al., 2008). The small leucine-rich proteoglycans (SLRPs), such as decorin, biglycan, fibromodulin, lumican, and osteoadherin, have also been identified in predentin and dentin (Goldberg et al., 1987, 2003a). Although not specific to dentin and can be found in other mineralized or non-mineralized tissues, they have been implicated in dentin formation and mineralization (Embery et al., 2001). They are thought to be involved in the transport of collagen fibrils through the predentin and in collagen fibrillogenesis (Goldberg et al., 2003a). Predentin is also rich in dermatan and chondroitin sulphate-containing (PG). It is of interest that adjacent to the mineralization front, predentin contains a large quantity of keratan sulphate-containing PG associated with a dramatic decrease in dermatan and chondroitin sulphate-containing PG. This switch in the proteoglycan type was attributed to MMP-3, which is closely related to a control of the dentin mineralization process (Hall et al., 1999).

Dentin also contains α-2-Heremans Schmid-glycoprotein (alpha-2-HSglycoprotein, AHSG), a serum protein currently known as Fetuin-A. Fetuin-A is a serum protein produced in the liver that concentrates in mineralized tissues, especially dentin because of its high affinity for hydroxyapatite (Mazzoni et al., 2012). The role for fetuins is to inhibit undesirable ectopic calcification without affecting normal bone or dentin mineralization. Interestingly, Fetuin-A can differentially control MMP-2 and -9 activities, both as an inhibitor, activator or stabilizer of these enzymes, depending on the enzyme and the time of binding (pro or activated form) (Mazzoni et al., 2012).

MMPs IN NON-CARIOUS DENTIN

MMPs are believed to play an important role in the matrix remodeling that takes place during dentinogenesis. The main MMPs identified in pulp, odontoblasts and in the predentin/dentin compartments are the collagenase MMP-8, the gelatinases MMP-2 and MMP-9, stromelysin-1 (MMP-3 or proteoglycanase), the MMP-2 activator MMP-14 (MT1-MMP), MMP-13, and enamelysin (MMP-20) (Palosaari et al., 2002; Sulkala et al., 2002, 2004, 2007; Goldberg et al., 2003b; Bourd-Boittin et al., 2004; Boukpessi et al., 2008; Mazzoni et al., 2009, 2011). TIMPs, their endogenous inhibitors were also detected but at a lower level of expression (Goldberg et al., 2003b; Palosaari et al., 2003). The expression of MMP-2, the predominant MMP in sound dentin, was shown to increase gradually from the onset of dentinogenesis to reach maximal expression at day 6–7 post-natal (Bourd-Boittin et al., 2005). MMP-2 is thought to play a key role in basement membrane degradation which allows a direct epithelio-mesenchymal contact, a prerequisite for odontoblasts and ameloblasts terminal cytodifferentiation (Sahlberg et al., 1992; Heikinheimo and Salo, 1995; Sahlberg et al., 1999). At the more advanced stages of dentinogenesis, MMP-2 and MMP-9 were shown to be located near the dentino-enamel junction (Goldberg et al., 2002, 2003b), and a strong gelatinase activity was detected by in situ zymography along the mantle dentin (Pessoa et al., 2013). MMP-2 was shown to cooperate with MMP-20 in the processing of dental ECM components, and particularly in that of DSPP (Bourd-Boittin et al., 2004; Yamakoshi et al., 2006), or in the self-processing mechanism of DSPP (Godovikova and Ritchie, 2007). Importantly, MMP-2 was isolated from mature human mineralized dentin matrix (Martin-De Las Heras et al., 2000) and zymographically identified in demineralized dentin (Van Strijp et al., 2003; Mazzoni et al., 2007), suggesting a potential role in dentin ECM degradation during the carious process (Tjaderhane et al., 1998; Chaussain-Miller et al., 2006). Recent

studies described a differential profile of localization and activity of the gelatinases in the different layers of human sound dentin (Boushell et al., 2011; Niu et al., 2011). High levels of MMP-2 were observed in odontoblasts where it was co-localized with TIMP-2. It was also observed in the deep dentine and at the dentinoenamel junction (Goldberg et al., 2003b). MMP-9, which colocalized with TIMP-1, was also shown to decrease from the deep to the superficial dentine layer (Niu et al., 2011). This gelatinase gradient may determine the rate of collagen degradation in pathological conditions, depending on the depth of the affected dentine.

Stromelysin-1 (MMP-3) has been identified in predentin, where it was proposed to participate in the mineralization process by degrading CS/DS (PG) (Hall et al., 1999) and in dentin where it was shown localized within the intertubular dentine, along the collagen fibrils (Mazzoni et al., 2011). Our own studies have shown that this enzyme is present in demineralized dentin in its active form, implying that it has the potential to degrade and disorganize the dentin matrix. Indeed, we have shown that when active, MMP-3 is able to cleave from the collagen scaffold several matrix proteins such as decorin, biglycan as well as four members of the SIBLING family: DSP, matrix extra-cellular phosphoglycoprotein (MEPE), bone sialoprotein (BSP) and osteopontin (OPN) (Boukpessi et al., 2008). The release by MMP-3 of (PG), such as decorin, could result in a subsequent release of the sequestered cytokines which in turn may activate other MMPs, thus potentiating the degradation of the demineralized matrix (Imai et al., 1997; Suppa et al., 2006).

POTENTIAL MECHANISM(S) OF MMP ACTIVATION IN CARIOUS DENTIN

MMPs are secreted to the ECM as inactive proenzymes and then require activation in order to be able to degrade matrix components. During the caries process, the acidic environment created by the release of bacterial acids can favor the activation of endogenous MMPs. Low pH was suggested to cause a conformational change within the propeptide domain of the enzyme that facilitates the cysteine switch, a critical step in the activation process (Tjaderhane et al., 1998). However, although the activated MMPs are stable in acidic pH, they can only be functional in neutral pH. Neutralization of the acids can be achieved by the dentinal buffering mechanisms (Camps and Pashley, 2000; Haapasalo et al., 2007) through the salivary buffer systems, thus allowing the pH-activated MMPs to cleave matrix components (Tjaderhane et al., 1998). In addition, the phosphorylated proteins released from the collagen scaffold by bacterial acids could interact with TIMP-inhibited MMPs within the carious lesion and re-activate them, enhancing the degradation process (Fedarko et al., 2004). Another enzyme family, the cysteine cathepsins, was identified in dentin (Tersariol et al., 2010) and in saliva of patients with periodontal conditions. They have also been reported in human carious dentin (Van Strijp et al., 2003) and it was suggested that these enzymes, when active, can in turn activate latent MMPs (Van Strijp et al., 2003). Along this line, a recent study reported a stronger immunostaining of cathepsins B in carious dentin, observed in dentin tubules and in odontoblasts, when compared with sound dentin (Nascimento et al., 2011). In addition, carious dentin displayed increased cysteine cathepsin activity, which was even greater in deep lesions with pulp exposure. Interestingly, it was also shown in this study that MMP activity in the saliva was higher in patients with active compared to chronic carious lesions, while salivary cathepsins did not show respective correlation. The reason may be that saliva is rich in cystatins (Dickinson, 2002), potent cysteine peptidase inhibitors. On the other hand, cathepsin activity increases with increasing depth of the lesion, indicating the role of pulp tissue-derived enzymes (Nascimento et al., 2011). Taken together, these studies suggest that cysteine cathepsins may participate in the carious process at least partly by activating latent MMPs.

DENTIN MATRIX DEGRADATION DURING THE CARIOUS PROCESS

Host MMPs derived from dentin or saliva have been shown to be able to degrade the dentin matrix which has been previously demineralized by bacterial acids (Tjaderhane et al., 1999; Chaussain-Miller et al., 2006) (**Figure. 1**). Saliva contains several MMPs including collagenases and gelatinases derived from either the gingival crevicular fluid or the secretion of salivary glands, MMP-9 being the most abundant as it is derived from both sources (Van Strijp et al., 2003). Indeed, the incubation of demineralized dentin slabs with acid-pretreated saliva resulted in the degradation of the organic matrix (Van Strijp et al., 2003). As saliva bathes the carious lesions, it is not surprising that the active form of MMP-9 was systematically detected by zymography performed on dentin extracts from several carious teeth (Tjaderhane et al., 1998). Together these studies indicate that salivary MMPs may have a strong contribution to dentin matrix degradation during the caries process. Carious lesions were also found to contain both latent and active forms of MMP-3, MMP-2 and MMP-8 (Tjaderhane et al., 1998; Mazzoni et al., 2007; Sulkala et al., 2007; Boukpessi et al., 2008). Along this line, dentin protein extracts obtained from the different dentin layers of decayed teeth were shown to have their gelatinase activity gradually increased from healthy dentin extracts to soft infected dentin extracts (superficial soft carious lesion, inner soft carious lesion, affected dentin, sound dentin) (Charadram et al., 2012). These observations confirm that endogenous MMP-2 contained within the sound dentin is activated during the carious process (**Figure. 2**). It has also been suggested by immunohistochemical observations that the endogenous MMP-2 level may be increased through the induction of MMP-2 synthesis by the presence of caries (Toledano et al., 2010). This was supported by a study showing that MMP-2 and TIMP-2 gene expressions were significantly up-regulated in odontoblasts adjacent to the carious lesion (Charadram et al., 2012). Interestingly, several studies have reported that in the cancer context, MMP expression by tumor cells was up-regulated by an acidic extracellular pH (Kato et al., 2005; Rofstad et al., 2006). This can suggest that acidic pH during the carious process may both induce MMP expression by the odontoblasts and favor their activation, potentiating MMP proteolytic capacity. In addition, the expression of MT1-MMP, a potent activator of MMP-2 and MMP-20 (Palosaari et al., 2002), was also dramatically increased in these cells, enhancing dentin matrix degradation. In addition, MMP-2, MMP- 20 and

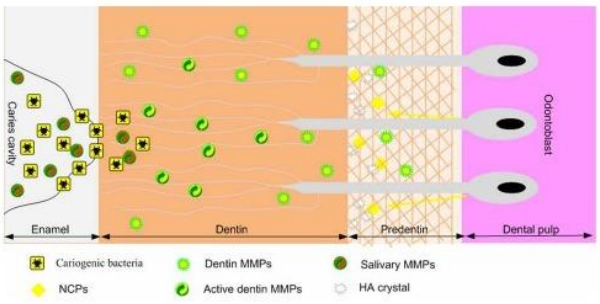

FIGURE 1 | Schematic representation of MMP activity during the dentin carious process. Cariogenic bacteria present in the caries cavity release acids such as lactic acid that reduce the local pH. The resulting acidic environment demineralizes the dentin matrix and induces the activation of host MMPs derived from dentin or saliva (which bathes the caries cavity). Once the local pH is neutralized by salivary buffer systems, activated MMPs degrade the demineralized dentin matrix.

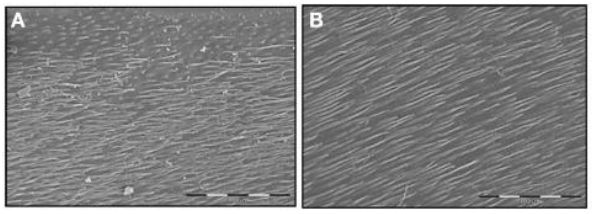

FIGURE 2 | SEM examination of the effects of MMP-2 on the dentin matrix through low viscosity resin replicas. (A) Standardized human dentin cubes were treated for 24 h with 0.5 M lactic (pH 4.8) and resin penetration in demineralized dentin was observed by resin replicas. The low viscosity resin penetrates in the main dentin tubules. **(B)** The treatment of demineralized dentin cubes by activated recombinant MMP-2 shows a deeper resin penetration into the main dentin tubules, suggesting increased dentin matrix degradation. Bar = 100 mm.

cathepsin B were shown to be present in dentinal fluid, where they may contribute to peritubular dentin degradation especially in young patients who have large and numerous dentin tubules (Sulkala et al., 2002; Boushell et al., 2008; Tersariol et al., 2010). Importantly, a study performed in a rat caries model has shown that MMP inhibition by several synthetic inhibitors reduced dentin caries progression under fissures (Sulkala et al., 2001). This study not only confirms the role of host MMPs in the caries process but also raises the possibility of using MMP inhibitors for impairing dentin matrix degradation during the caries.

MMP INHIBITORS FOR THE TREATMENT OF CARIOUS DENTIN

Considering the potential role of MMPs in dentin ECM degradation, it seems logical that, when associated with a thorough control of the caries (Selwitz et al., 2007), MMP inhibition would help to control dentin caries progression. This notion was supported by studies in rat caries models where dentin caries progression was delayed by intra-oral administration of chemical MMP inhibitors (Tjaderhane et al., 1999; Sulkala et al., 2001). Several effective MMP inhibitors have been described. Tetracyclines and their derivatives, doxicycline and minocycline are able to inhibit MMP activity independent of their antimicrobial action. They are commonly used as antibiotics in the treatment of periodontitis (Ryan et al., 1996) and have been shown both *in vitro* and *in vivo* to inhibit MMP-1, MMP-2, MMP-8 and MMP-12 (Golub et al., 1995; Lauhio et al., 1995). Another effective and safe MMP inhibitor is the non-antimicrobial chemically modified tetracyclines (CMTs), which can inhibit both the release and the activity of MMPs (Golub et al., 1998; Ramamurthy et al., 1998). Zoledronate is a third generation bisphosphonate which has the ability to inhibit MMP proteolytic activities (Teronen et al., 1997; Boissier et al., 2000). Sulkala et al. (2001) have shown that the systemic MMP inhibition with CMT-3 and zoledronate *in vivo* suppressed the progression of dental caries under fissures, indicating that systemic administration of MMPs inhibitors could be effective in caries prevention (Sulkala et al., 2001). Indeed, the authors observed a reduction in the progression of caries in rats treated with these MMP inhibitors, though no synergistic potentiating effect of these two compounds could been demonstrated.

In humans, MMP inhibitors would preferably be administered locally to treat dental caries by either incorporating them in topical preparations for daily use or by applying them directly on the dentin surface, depending on the clinical situation. When treating coronal caries, especially in young patients with deep and active caries lesions, a solution containing MMP inhibitors may be applied directly to the lesion after the mechanical removal of the caries and before restoration. A second strategy would be to incorporate these inhibitors in mouth rinses or toothpastes to prevent root caries progression. Several synthetic MMP inhibitors are already used in the dental practice. The MMP inhibitory action of most of them is based on their zinc-/calcium-chelating groups, since MMPs require metal ions (calcium and zinc) for their catalytic activity (Gendron et al., 1999). Among them, Ethylenediaminetetraacetic acid (EDTA), which is an effective zinc and calcium chelator, was recently shown to inhibit the degradation by acid-activated endogenous MMPs of dentin beams treated for 1 min with 17% EDTA (Thompson et al., 2012). Chlorhexidine digluconate (CHX) has also potent MMP inhibitor effects that also involve a calcium-chelating mechanism (Gendron et al., 1999). These inhibitors have been shown to improve the integrity of the hybrid layers obtained by a simplified etch-and-rinse adhesive after dentin caries removal (Carrilho et al., 2007). The local application of 2% CHX for 1 min to the etched dentin surface just before applying the dentin bonding primer was able to inhibit the degradation of the hybrid layer by MMPs for at least 14 months. This clinical study highlights that CHX, a MMP inhibitor already used in dental practice, is able to impair dentin matrix degradation. Interestingly, Scaffa et al. demonstrated that CHX was also a potent inhibitor of the cysteine cathepsin enzymes (Scaffa et al., 2012), which were shown to be present and active in sound and carious dentin (Tersariol et al., 2010; Nascimento et al., 2011).

A different group of MMP inhibitors include those derived from natural sources. Green tea polyphenols, especially

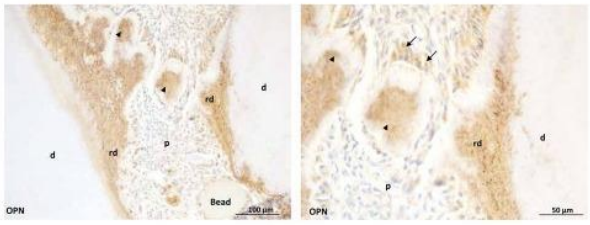

FIGURE 3 | Early dentin repair in an injured rat pulp model induced by the implantation of C-DMP1 peptide mimicking an MMP2 cleaved peptide of DMP1.Beads soaked with c-DMP1 polypeptide were implanted in the injured pulp of young rats. At day 7, osteopontin (OPN) immunohistochemistry shows strong immunoreactivity of reparative structures (arrowheads) observed in the injured pulp resembling mineralization foci (Mckee et al., 1993). Bridge formation at longer time points may partly result from growth and spreading of these mineralization foci. At higher magnification (right panel), immunopositive cells (arrows) are observed adjacent to the reparative structures.p: pulp; rd: reactionary dentin; d: dentin.

epigallocatechin gallate (EGCG), were found to have potent and distinct inhibitory activity against MT1-MMP, resulting in the decrease of MMP-2 activation. Furthermore, EGCG inhibits directly MMP-2 and MMP-9 (Demeule et al., 2000; Garbisa et al., 2001; Dell'aica et al., 2002), and was recently shown to inhibit dentinal erosion, along with other known MMP inhibitors (Kato et al., 2010). Grape seed extract (GSE) has been shown to suppress lipopolysaccharide-induced MMP secretion by macrophages and to inhibit MMP-1 and MMP-9 activity in periodontitis (La et al., 2009a). Recent *in vitro* studies demonstrated that GSE inhibited the demineralization and/or promoted the remineralization of artificial root carious lesions under dynamic pH- cycling conditions (Xie et al., 2008; Pavan et al., 2011). The MMP-inhibitory effects of these or other natural substances such as cranberry proanthocyanidins (La et al., 2009b) suggest that they could be effective in slowing down dentin caries progression. The fact that these molecules are devoid of undesirable side effects, when compared with those of synthetic drugs, makes them particularly attractive for the treatment of dentin caries, since they can be safely applied directly on the affected tooth or integrated in daily-used topical products.

GENERATION OF BIOACTIVE PEPTIDES FROM DENTIN MATRIX DEGRADATION

By cleaving large insoluble ECM components, MMPs are known to liberate bioactive fragments and cytokines (Mott and Werb, 2004). The release of active peptides by the enzymatic cleavage of dentin matrix proteins by MMPs during the carious process was also suggested (Chaussain-Miller et al., 2006). These peptides, which often retain the activity of the parent protein (Dean and Overall, 2007), or display a function different from those of the protein they are derived from, can affect several biological properties in terms of proliferation, angiogenesis, differentiation that may favor the pulp healing process (Dean et al., 2007; Smith et al., 2012a). In particular, the degradation of matrix (PG), which often bind and sequester growth factors, by proteases such as the MMP-3 would cause the release and activation of these factors within the carious lesion and can as a result impact pulp healing (Smith et al., 2012b). The gelatinase MMP-2 has been proposed to be able to release active TGF-β2 in the carious lesion, which can then stimulate diverse repair processes (Charadram et al., 2012).

Using an injured rat pulp model to test the biological properties of such cleavage products derived from the (ECM), we demonstrated that a polypeptide containing the C-terminal part of DMP1, and mimicking DMP1 cleavage product by MMP-2, was able to enhance pulp healing capacity when implanted in a rat pulp-injury model (Chaussain et al., 2009). A dense and continuous reparative dentin bridge was rapidly observed after implantation and the cells boarding the bridge presented a characteristic polarized odontoblastic phenotype, organized as a palisade and expressing the odontoblastic markers DSP and DMP1 (**Figure 3**). The use of such ECM-derived bioactive peptides associated with restorative materials constitutes a promising therapeutical option for tooth repair (Goldberg et al., 2008). In addition, these peptides may also be used for bone regeneration, potentially associated with engineered scaffolds and mesenchymal stem cells (Mao et al., 2006), as dentin and bone are similar in several aspects, especially pertaining to the composition of the ECM (Opsahl Vital et al., 2012).

In conclusion, accumulating data have demonstrated that endogenous enzymes contained in dentin and saliva are involved in the dentin caries process. These findings are important, since they open new options for caries prevention and treatment. Slowing down or preventing the irreversible destruction of the organic matrix would allow for natural healing of the lesion by remineralization. MMP inhibitors may prove to be useful in the prevention of dentin caries progression. However, the outcomes of this approach will depend on the management of the caries risk, involving the control of caries risk factors such as cariogenic biofilm, diet, inadequate salivary flow rate and insufficient fluoride exposure (Selwitz et al., 2007).

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3 Results

3.1 Role of EMMPRIN in tooth formation

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Original Full Length Article

EMMPRIN/CD147 deficiency disturbs ameloblast–odontoblast cross-talk and delays enamel mineralization

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ABSTRACT

Tooth development is regulated by a series of reciprocal inductive signaling between the dental epithelium and mesenchyme, which culminates with the formation of dentin and enamel. EMMPRIN/CD147 is an *E*xtracellular *M*atrix *M*etallo*P*Roteinase (MMP) *IN*ducer that mediates epithelial–mesenchymal interactions in cancer and other pathological processes and is expressed in developing teeth. Here we used EMMPRIN knockout (KO) mice to determine the functional role of EMMPRIN on dental tissue formation. We report a delay in enamel deposition and formation that is clearly distinguishable in the growing incisor and associated with a significant reduction of MMP-3 and MMP-20 expression in tooth germs of KO mice. Insufficient basement membrane degradation is evidenced by a persistent laminin immunostaining, resulting in a delay of both odontoblast and ameloblast differentiation. Consequently, enamel volume and thickness are decreased in adult mutant teeth but enamel maturation and tooth morphology are normal, as shown by micro-computed tomographic (micro-CT), nanoindentation, and scanning electron microscope analyses. In addition, the dentino-enamel junction appears as a rough calcified layer of approximately 10 ± 5 μm thick (mean ± SD) in both molars and growing incisors of KO adult mice. These results indicate that EMMPRIN is involved in the epithelial-mesenchymal cross-talk during tooth development by regulating the expression of MMPs. The mild tooth phenotype observed in EMMPRIN KO mice suggests that the direct effect of EMMPRIN may be limited to a short time window, comprised between basement membrane degradation allowing direct cell contact and calcified matrix deposition.

Introduction

Tooth development results from reciprocal inductive interactions between the ectomesenchyme and oral epithelium and proceeds through a series of well-defined stages including the initiation, bud, cap and bell stages [1–6]. At the bell stage which is the last step of tooth crown formation, signals from the dental epithelium (*i.e.*, inner enamel epithelium) instruct dental mesenchymal cells to differentiate into odontoblasts. Differentiated odontoblasts signal back to inner enamel epithelial cells and induce their differentiation into ameloblasts, which are responsible for enamel matrix synthesis. Ameloblast terminal differentiation necessitates the presence of an extracellular matrix that is secreted by odontoblasts and forms the predentin [7]. The degradation of the basement membrane (BM) separating the dental epithelium from the mesenchyme is a key step in this process that allows direct contact of ameloblasts with both odontoblasts and the unmineralized dentin matrix [3,8]. Matrix metalloproteinases (MMPs) are involved in all stages of tooth formation [9–11]. At the bell stage, MMPs have a major role in BM degradation [12,13], thus allowing direct cross-talk between odontoblasts and ameloblasts [12–14]. It has been shown that at more advanced stages MMPs also regulate the processing of dental extracellular matrix (ECM) proteins prior to mineralization. Indeed, it has been demonstrated that MMPs regulate amelogenin (AMEL) cleavage by enamelysin (MMP-20) during early enamel maturation [9,15–20].

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The notion of direct epithelial–mesenchymal (or epithelio–stromal) interactions was first introduced in the cancer field when EMMPRIN, a membrane glycoprotein also known as CD147, was identified as a MMP inducer present at the cell surface of tumor cells which can activate stromal cells through direct contact and signal them to increase MMP production [21]. Recently accumulating data also advocate a role for EMMPRIN in modulating MMP expression during non-tumorigenic pathological conditions as well as in physiological situations such as tissue remodeling and cytodifferentiation events [22–29]. For example, the presence of EMMPRIN in tissues affected by rheumatoid arthritis indicates that it is also involved in bone and cartilage deterioration [30–32]. Furthermore, it has been suggested that EMMPRIN plays a role in cell differentiation, polarity and survival [33]. Knockout (KO) mice have been generated to analyze the functional significance of EMMPRIN during development and adulthood [34]. These mice exhibited several developmental defects, are smaller than wild-type (WT) mice and are usually unable to undergo implantation. The EMMPRIN mutant offspring are sterile, thus demonstrating deficiencies in spermatogenesis and fertilization. In addition, these null mice are blind due to defective retinal development and have faulty sensory and memory functions [34–36].

The expression of EMMPRIN in the developing tooth germs was previously described [37,38]. EMMPRIN expression was shown to increase gradually in the forming molar germ in mice from E14 to P1 [38]. Expression was also reported in tooth germs of rats at the bell stage, in the enamel organ on the cell membranes of ameloblasts and in the stratum intermedium, as well as in the mesenchyme at the distal ends of differentiating odontoblasts [37]. Using *ex-vivo* culture of E11 mice mandibles treated with EMMPRIN siRNA, Xie et al. have proposed that EMMPRIN may be involved in tooth morphogenesis [38]. However, the *in vivo* role of EMMPRIN in tooth development and homeostasis is still unknown. In this study, our objective was to investigate the EMMPRIN functions in tooth formation using EMMPRIN KO mice by exploring the modifications occurring in their dental phenotype and the consequences on EMMPRIN's molecular targets, in particular on MMPs. The expression of the gelatinases (MMP-2 and MMP-9), stromelysin-1 (MMP-3), and enamelysin (MMP-20) was more particularly investigated as well as their possible role in basement membrane degradation and early enamel maturation, two critical steps for normal tooth formation where MMP activity has been shown to be important [12,13,39,40]. Our results suggest a role for EMMPRIN in epithelial–mesenchymal interactions by regulating the expression of specific MMPs.

Material and methods

Animals

EMMPRIN mice were a kind gift from Dr. Takashi Muramatsu, Department of Biochemistry, Nagoya University School of Medicine, Japan [34]. Heterozygous breeding was carried out and tail snips were collected for genotyping. DNA was extracted from the snips using DNeasy Blood and Tissue Kit (Qiagen, France) and the genotype was determined by PCR using primers for EMMPRIN and Neomycin resistance genes [34]. Offspring with the EMMPRIN gene null mutant (KO) phenotype were maintained under the same conditions as those for the wild type (WT) mice. Post-natal littermate WT (+/+) and KO (−/−) mice at day 1, day 5, 3 and 18 months were examined in this study. The protocol was approved by the Animal Care Committee of French Veterinary Services (DDSV Val de Marne, France).

Immunohistochemistry

The heads of post-natal day 1 WT and KO mice were fixed overnight in a 4% paraformaldehyde solution (6 mice per group). The samples were progressively dehydrated and embedded in Paraplast (Oxford Labware, St Louis, MO, USA). Seven-micrometer-thick tissue sections were prepared, deparaffined and rehydrated. Endogenous peroxidases were blocked in 0.4% hydrogen peroxide in methanol. After extended rinsing in PBS, background activity was blocked at room temperature for 90 min in 1% bovine serum albumin (BSA) in PBS. Goat polyclonal EMMPRIN antibody (Santa Cruz, Heidelberg, Germany), rabbit monoclonal MMP-20 antibody (Abcam, Cambridge, UK), rabbit polyclonal laminin antibody (Sigma-Aldrich, Saint-Louis, MO) and rabbit polyclonal amelogenin antibody (kind gift to EA2496 from Dr. Carolyn W. Gibson, Department of Anatomy and Cell Biology, University of Pennsylvania School of Dental Medicine, Philadelphia, PA) were used at 1/400, 1/100, 1/100 and 1/150 dilution, respectively. Sections were treated overnight in a moist chamber at 4 °C and further incubated for 90 min with a polyclonal swine anti-rabbit immunoglobulin-G peroxidase-conjugated or a polyclonal rabbit anti-Goat immunoglobulin-G peroxidase-conjugated (Dako, Glostrup, Denmark), as secondary antibody. Peroxidase labeling was revealed (1 to 10 min according to the antibody) in a dark chamber using 3–3 diaminobenzidine tetrahydrochloride (Sigma-Aldrich) with hydrogen peroxide in Phosphate Buffered Saline solution (PBS). To exclude nonspecific binding, controls were carried out by omitting the primary antibody. For laminin, a goat anti-rabbit immunoglobulin-G Alexa Fluor 488 was used at 1/200 (Sigma-Aldrich) in a dark-chamber and nuclei were stained with DAPI solution at 1/4000 (Invitrogen, Life technologies, Carlsbad, CA) for 3 min. Images were merged using Image J software (NIH, Bethesda, MD).

Total protein extraction from tooth germs

Maxillary and mandibular molar germs (M1 + M2) were dissected from 5 day-old post-natal WT and KO mice (n = 6 mice per group). Tooth germs were mixed and ground to a fine powder in the presence of liquid nitrogen, then homogenized in ice-cold extraction buffer (10 germs in 200 μL buffer: 50 mM Tris–HCl, pH 7.5, containing 5 mM EDTA, 0.9% NaCl and 0.2% Triton X-100), supplemented with 1/100 Protease Inhibitor Cocktail Set V EDTA free (Calbiochem, La Jolla, CA) [9]. The resulting homogenates were then briefly sonicated on ice (3 times 5 s), cleared by centrifugation at 10,000 *g* for 15 min at 4 °C and stored at −80 °C. Same procedure was carried out with the soft part of the growing lower incisor from 3 month-old mice.

MMP activity measurement

MMP activity was measured using the generic-fluorescent quenched substrate Mca-PLGL-(Dpa)-AR-NH2 (R&D System, Minneapolis, MN). Briefly, one microgram of each tooth germ extract was incubated with 2 μM Mca-PLGL-(Dpa)-AR-NH_2 in a 50 mM Tris–HCl buffer, pH 7.5, containing 150 mM NaCl, 5 mM $CaCl_2$ and 0.05% Brij 35. The reaction was allowed to proceed overnight at 22 °C and then was stopped by adding 10 mM EDTA. The rate of substrate cleavage was measured in triplicate for each extract using a Tecan Infinite® M1000 spectrofluorometer with excitation and emission wavelengths of 326 and 393 nm, respectively. Less than 5% of the substrate was hydrolyzed during the rate measurements.

Zymographic analysis

Protein homogenates were analyzed by gelatin zymography to identify MMP-2 and MMP-9 isoforms. Electrophoresis was carried out using a miniprotean II system (BIO-RAD, Hercules, CA) on 8% polyacrylamide gels containing 1 mg/ml gelatin. Samples (3 μg) were half-diluted in 1 M Tris, pH 6.8, containing 50% glycerol and 0.4% bromophenol blue, and gels were run under Laemmli conditions. Following electrophoresis gels were washed twice (30 min each) in 200 ml 2.5% Triton X-100 under constant mechanical stirring and incubated in 50 mM Tris–HCl buffer, pH 7.5, containing 150 mM NaCl, 5 mM $CaCl_2$ and 0.05% Brij 35, for 24 h at 37 °C. Gels were stained with 0.25%

Coomassie brilliant blue G-250 (50% methanol, 10% acetic acid) and destained appropriately (40% methanol, 10% acetic acid). Proteinase activity was evidenced as cleared (white) bands. Bands were digitalized and compared using image J software. Homogenates were also analyzed by casein zymography to identify MMP-20 and MMP-3. Zymograms were run on Novex 4–16% casein zymogels (Life Technologies, Carlsbad, CA), incubated at 37 °C for 48 h in 50 mM Tris–HCl buffer, pH 7.5, containing 5 mM $CaCl_2$ to allow MMP activity. To detect KLK-4, casein zymograms were run in the same conditions except that 20 mM EDTA was added to the incubation buffer to inhibit MMP activity (adapted from [40]). For casein zymograms, recombinant MMP-3 (R&D system) and MMP-20 (kind gift from Pr Pamela K. DenBesten, UCSF, CA) were loaded as positive control.

Western blot analysis

Protein homogenates (20 μg) were subjected to SDS-PAGE on 4–20% precast gels (Mini-PROTEAN TGX Precast Gels, BIO-RAD) and transferred onto a nitrocellulose membrane (BIO-RAD). Membranes were incubated with anti-amelogenin (1/750), anti-enamelin (1/500, Santa Cruz), anti-ameloblastin (1/1000, R&D system), anti-EMMPRIN (1/1000), anti-MM-20 (1/1000, N-terminal MMP-20 antibody, Sigma, Saint Louis, MO), anti-KLK4 (1/500, Santa Cruz), or anti-MMP-3 (1/700; Abcam) antibodies overnight at 4 °C. The membranes were incubated with a 1/1000 dilution of a peroxidase-linked anti-rabbit IgG (amelogenin, MMP-3 and −20, KLK4) or anti-goat (EMMPRIN, enamelin and ameloblastin) secondary antibody for 1 h at room temperature and developed by means of BM Chemiluminescence Western Blotting Kit (Roche Diagnostics, Meylan, France). As a control for protein loading, the membranes were carefully washed and stripped with stripping buffer (Pierce Chemical, Rockford, IL) and processed with a rabbit monoclonal anti-GAPDH antibody (EPITOMICS, California, USA). The relative intensity of bands was digitalized and quantified using image J software.

Micro-X-ray computed tomography (micro-CT) analysis

For exploration of potential mineral part of tooth alterations in adult mice, 3 and 18 month-old mandibles (6 mice per group) were fixed for 48 h in 4% paraformaldehyde and stored in 70% ethanol at 4 °C. Half mandibles were subjected to a desktop micro-CT, (Skyscan 1172, Skyscan, Aartselaar, Belgium). The micro-CT settings were used as follows: 9 μm resolution, voltage 80 kV; current 100 μA; exposure time 400 ms; 180° rotation; rotation step 0.4°; and frame averaging 4. The scanning time was approximately 4 h/sample. A total of 1700 native slice frames per sample were reconstructed using NRecon software (Skyscan, Belgium). Total mineralized tissue volume (80–255 grayscale units) was compared to enamel volume using this software. The lower and upper thresholds for enamel were defined to be from 181 to 255 grayscale units, while the thresholds for bone and dentin were defined as 80–180 grayscale units on an 8-bit grayscale. Same values were used for all samples analyzed. The dicom data frames were subsequently analyzed using OsiriX imaging software (3.7.1 version). The scans resulted in reconstructed 3-D data sets with a voxel-size of 9.92 μm. Enamel thickness was measured in M1, M2, incisor under M1, and incisor under M2 from μCT images using CTAn software (Skyscan, Belgium) following a previously described method [41].

Nanoindentation

Cross-section samples of molars and incisors were prepared by cutting the resin-embedded samples perpendicular to the sagittal axis of the jaw, at the level of the mesial cusp of the first molar. In order to achieve an appropriate flatness and surface quality for the nanoindentation experiments, all surfaces of the cross-sections were grinded using a Presi Mecapol P230 polishing machine. For the grinding procedure SiC paper was applied starting with a mesh size of 120, which was subsequently decreased to 4000. After cleaning, the nanoindentation measurements were performed using a G200 Nanoindenter (Agilent Technologies, Inc., Vista, CA, USA) equipped with a Berkovich-shaped diamond indenter tip. Samples were tested in dry condition. For all nanoindentation measurements, the displacement-controlled continuous stiffness mode (csm) technique was applied. In this mode, small oscillations of the indenter tip are superposed to the overall indenter displacement of the loading and unloading testing cycle [42]. Using the csm mode allows determining material parameters such as hardness and Young's modulus values as a function of depth for the complete range of indentation. For the experiments a maximum indentation depth of 3000 nm was chosen while the loading speed was set to result in a strain rate (averaged within the locally loaded sample region) of 0.05 1/s. Typically, 15 single indentation tests were performed for each of the specimen regions to be investigated (e — enamel; dfp — dentin far from pulp; dmr — dentin middle region; *cf.* the light microscopic image in Fig. 4C). A representative indentation depth of 500 nm was chosen for determining the nanoindentation data on hardness and Young's modulus. This value was found well suitable to compare all different enamel and dentine samples while minimizing the effects resulting from surface microstructure. The measured modulus and hardness data are in close agreement with the values found in similar nanoindentation studies of mice molars [43,44] determined at comparable indentation depths.

SEM analysis

Mandibles of the 3 month-old mice were prepared for SEM analysis. Cross-sections at the level of the mesial cusp of the first molar were performed with a saw equipped with a diamond disk (Struers, Champigny sur Marne, France) under a continuous water spray. After thorough polishing, surfaces were cleaned with 5% sodium hypochlorite for 2 min, rinsed twice with distilled water, etched with 2% Nitric acid for 10 s, and then thoroughly rinsed with distilled water. Carbon or gold sputter-coated surfaces were observed under a scanning electron microscope (Cambridge S260 SEM, UK), equipped with an Everhart–Thornley and BSE electron detector, and EDX spectrometer for X-ray microanalysis (BRUKER AXS, France) with Esprit2 software.

RT-PCR analysis for gene expression

Gene expression was analyzed after extraction of total RNA from the latest 2 mm of mandibular incisor root (soft part) of 3-month-old EMMPRIN −/− and +/+ mice (n = 6 mice per group), using Mini RNA Isolation kit (GE Healthcare, Little Chalfont, United Kingdom). The integrity of RNA was controlled with a Bioanalyzer (Agilent Bioanalyzer), purity checking and RNA dosage were carried out with a nanodrop (Nanovue Plus, GE Healthcare Life Sciences, UK). 250 ng of total RNA was reverse-transcribed with (cDNA kit VersoTM, ThermoFicher, Waltham, MA). For semi-quantitative RT-PCR, cDNA was amplified with specific primer (Go Tag®Hot Start polymerase, Promega, Fitchburg, WI). *MMP-2*, *MMP-3*, *MMP-9*, *MMP-20*, *EMMPRIN*, and *KLK-4*, cDNA expression were compared to *GAPGH* expression (Supplementary Table 1).

For quantitative real-time PCR (qRT-PCR) analysis, cDNA was amplified with specific primers (Eurofins mwg operon, Ebersberg, Germany) in a Light cycler® 480 SYBR Green I Master (Roche, Penzberg, Upper Bavaria, Germany). Each primer pair was chosen for the best efficiency and controlled for specificity (Supplementary Table 1). qRT-PCR analysis was carried out using LigthCycler 480 [45]. The PCR cycles consisted of an initial pre-incubation step (95 °C for 5 min), followed by 45 cycles of amplification (95 °C for 5 s, 55 °C for 5 s, 70 °C for 10 s). Relative gene expression levels were estimated using the deltaCq method [46]. *GAPDH* and *ubiquitine C* (*UBC*) were determined using GeNorm software on a set of 5 standard housekeeping genes and were used as housekeeping

genes for the rest of the study. Results were expressed as mean ± SD of relative mRNA level for EMMPRIN −/− and +/+ mice.

Isolation and culture of ED18 tooth germs

C57B6 wild type mice were mated overnight and the detection of the vaginal plug was counted as day 0 (ED0). Embryos were collected from one pregnant mouse at day 18 (E18) and the mandibular first molar extracted from hemi-mandible under a stereoscopic microscope. The tooth germ was then cultured on a semi-solid medium made of DMEM/F12 containing 20% fetal calf serum (FCS) supplemented with ascorbic acid (0.18 mg/ml), L-glutamine (2 mM), penicillin/streptomycin (50 units/ml) and agar (0.36%) [47]. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO2.

Small interfering RNA transfection (siRNA)

Knockdown of EMMPRIN expression was performed on molar tooth germs by siRNA treatment one day after the beginning of the culture. Two different siRNA oligos, siEMMPRIN (Ambion, siRNA ID: 10372–siRNA ID: s2098, Applied Biosystems) or scramble siRNA oligos (BLOCK-iT fluorescent oligo, Invitrogen), siScramble, were transfected in the cultured germ in the absence of FCS and antibiotics. Briefly, siRNA was first diluted in Opti-MEM medium before an incubation of 15 min with the mix of Lipofectamine RNAiMAX transfection reagent previously diluted in Opti-MEM (Invitrogen). Each siRNA was added on the cultured germ at the final concentrations of 60 and 120 nM. After 48 h of incubation, transfection medium was removed and germs were treated for RNA extraction. This experiment was repeated in three independent experiments.

Statistical analysis

Values are presented as means ± SD with at least 6 samples (6 germs or 6 animals) per group. The normality of the distribution was tested with the Shapiro-Wilk test and the homogeneity of variance was tested with the F-test. When the distribution of the data for each group respected the normal law and the variance of both groups were homogeneous, *T*-test for non-paired measures was used. When the distribution of at least one of the groups did not follow the normal law, the U-Mann Whitney test was applied. The critical p-value for statistical significance was $p = 0.05$.

Results

Tooth phenotype in EMMPRIN KO mice

Tooth phenotype was examined on post-natal day 1 mandible in first (M1) and second (M2) molars (Fig. 1 panel A). At this time point, the first molar (M1) germ is at the late bell stage characterized by

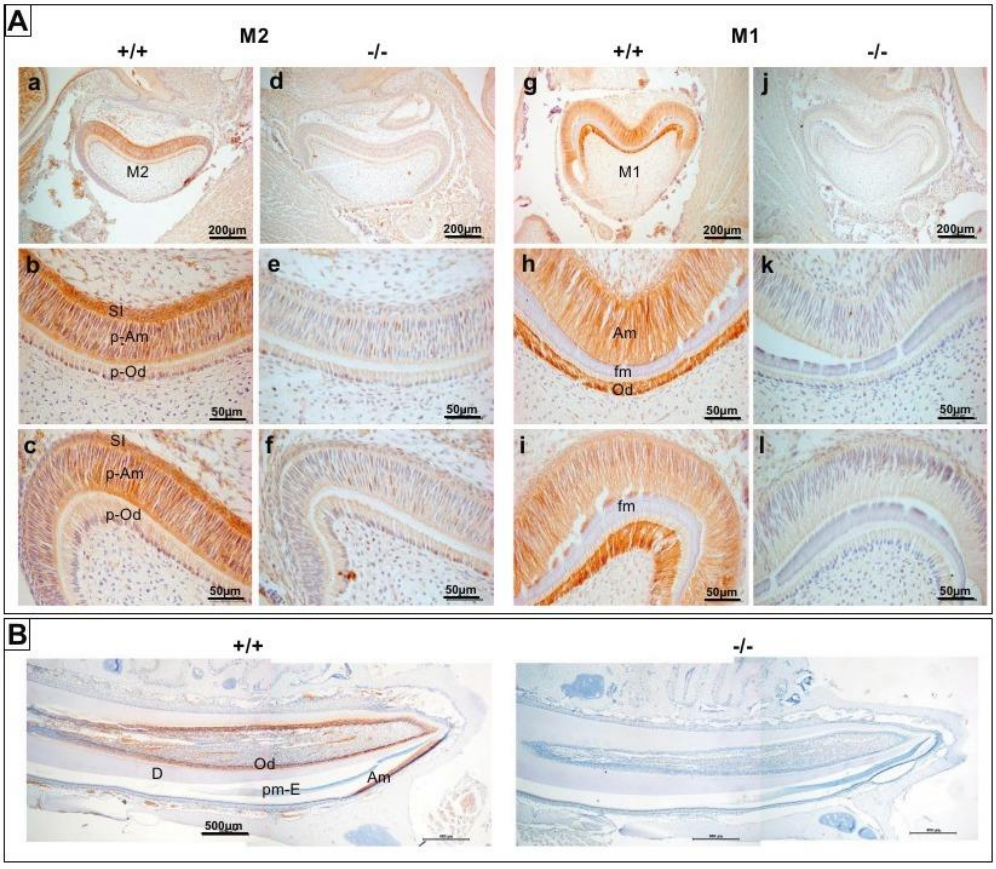

Fig. 1. Comparison of tooth germs in KO and WT mice. Panel A: frontal section of the mandible at post-natal day 1. No difference in the molar germ morphology is observed between WT (+/+) and KO (−/−) mice, either at early bell stage (M2) or at late bell stage (M1). KO mice show no EMMPRIN immunostaining (d–f, j–l). In WT mice, the pre-ameloblasts and stratum intermedium are positive for EMMPRIN in M2 germ (a). The staining is particularly intense in the intercuspal curve (b) and decreases at the tip of the cusps (c). At a later stage (M1 germ), when cells actively secrete protein matrix, a light staining is visualized on ameloblast cell membrane (g). This staining progressively decreases from the intercuspal curve (h) to the tip of the cusps (i). By contrast, an intense staining of the odontoblasts is observed (h, i). Panel B: sagittal section of 3-month-old mandible. No difference in the incisor morphology is observed between WT (+/+) and KO (−/−) mice. No EMMPRIN immunostaining is observed in KO, whereas both ameloblasts and odontoblasts are labeled in WT. p-Am: pre-ameloblast; p-Od: pre-odontoblast; Am: ameloblast; Od: odontoblast; Si: stratum intermedium; fm: forming matrix; D: dentin; pm-E: premature enamel.

terminal odontoblast and ameloblast differentiation. The second molar (M2) germ is at early bell stage characterized by last cell mitosis [8] and basement membrane degradation allowing for terminal cell differentiation. When examined microscopically, the morphology of the developing molar germs of WT and EMMPRIN KO mice appeared similar with well-visible formed tooth crowns (Fig. 1 panel A). EMMPRIN immunohistochemical analysis confirmed the absence of EMMPRIN expression in KO mice. In WT, EMMPRIN expression was seen in both ameloblasts and odontoblasts, depending on the development stage (Fig. 1, panel A). Such observations, including morphology and EMMPRIN expression were also made on sagittal sections of the developing incisor in 3-month-old mice, which as a continuously growing tooth, recapitulates all the developmental stages (Fig. 1, panel B, Supplementary Fig. 1).

Micro-CT analysis of mandibles from 3 month-old mice showed no evident difference between molars but a delay in mineralization of the continuously growing incisor in EMMPRIN KO mice (Fig. 2A). In WT mice, incisor mineralization was observed underneath M3, whereas mineralization was only visible at the level of M2 in KO mice. Quantitative analysis of reconstructed mandible samples demonstrated a significant lower enamel volume in EMMPRIN KO mice when compared to WT mice, $6.94.10^8$ μm^3 *versus* $10.85.10^8$ μm^3, respectively (Fig. 2B). Similar mineralization delay of the continuous growing incisor was observed in 18 month-old mice (data not shown). Enamel thickness, measured at M1, M2, incisor under M1, and incisor under M2 from Micro-CT images, was significantly lower in EMMPRIN KO mice compared to WT (0.139 ± 0.006 *versus* 0.117 ± 0.006 mm at M1, $p < 0.05$; 0.140 ± 0.003 *versus* 0.124 ± 0.001 mm at incisor under M1, $p < 0.001$; 0.148 ± 0.007 *versus* 0.112 ± 0.006 mm at M2, $p < 0.01$, and 0.142 ± 0.002 *versus* 0.127 ± 0.002 mm at incisor under M2, $p < 0.01$; respectively) (Fig. 2B).

To assess the quality of enamel and dentin associated with EMMPRIN deficiency, we then performed nanoindentation on 3 month- old samples in frontal sections performed at the level of M1 (Fig. 2C). No significant change of the enamel hardness and elasticity (Young's modulus) was observed between KO and WT mice both in M1 and in the underlying growing incisor. Furthermore, dentin analysis by nanoindentation showed no significant modification either in the outer or in the deeper dentin layer.

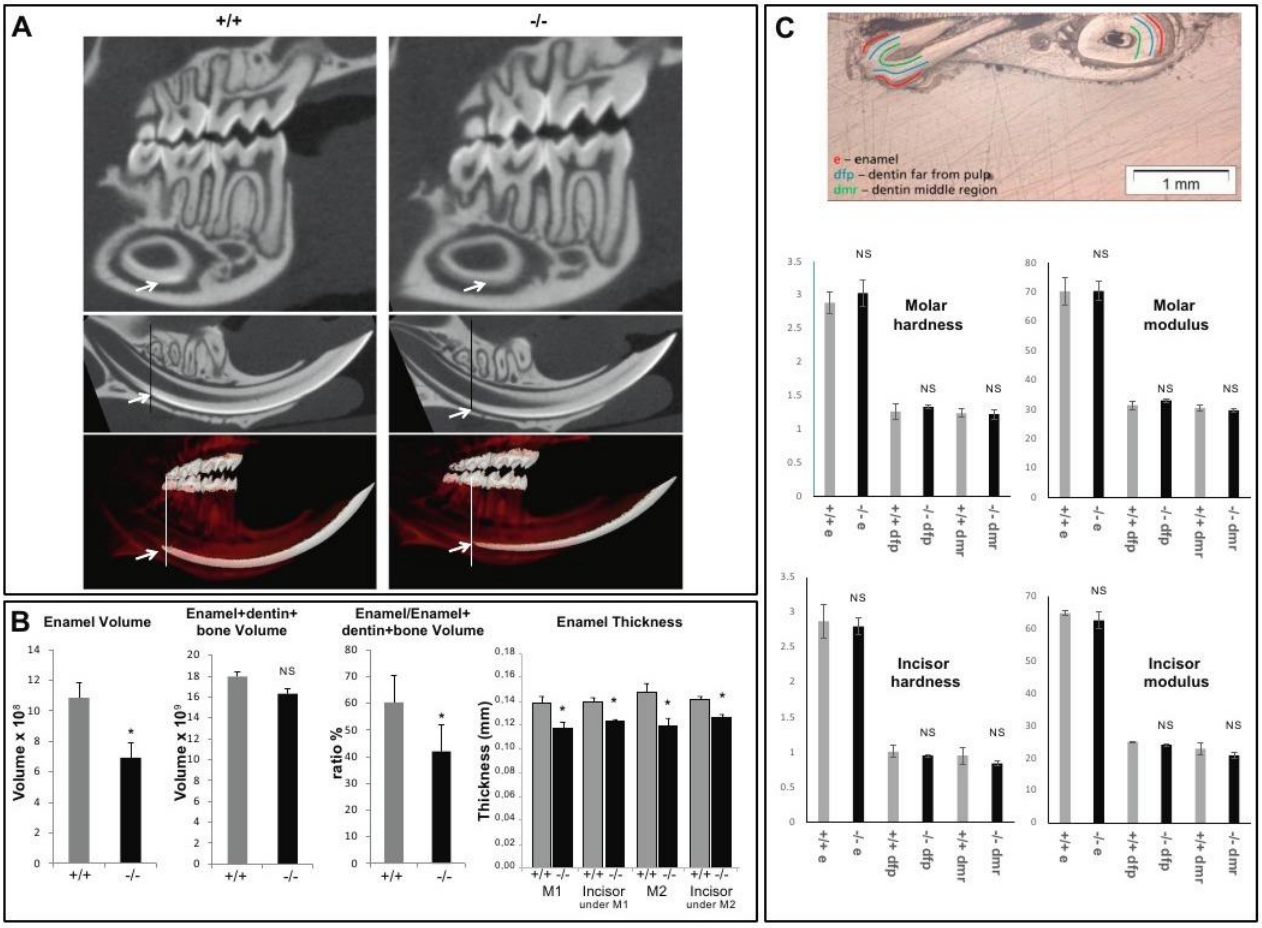

Fig. 2. Micro-CT and nanoindentation analysis of mandibles of EMMPRIN KO adult mice when compared with WT. Mandibles collected from 3 month-old mice were analyzed using micro-CT and nanoindentation. Panel A: reconstructed micro-CT data show a mineralization delay of the developing incisor (I, white arrow) in KO mice. In WT mice, incisor mineralization (white arrow) is observed under M3 whereas in KO it is detected under M2. Panel B: quantitative analysis of reconstructed mandible samples shows a significant lower enamel volume in EMMPRIN KO mice compared to WT ($6.94.10^8$ μm^3 *versus* $10.85.10^8$ μm^3, respectively). A non-significant (NS) decrease is observed for total mineralized tissue volumes ($16.34.10^9$ μm^3 *versus* $17.96.10^9$ μm^3, respectively). A 31% decrease in the ratio enamel volume/total mineralized tissue volume is seen in KO group. Enamel thickness, measured at M1, M2, incisor under M1 and incisor under M2 from μCT images, was significantly lower in EMMPRIN KO mice compared to WT (0.139 ± 0.006 *versus* 0.117 ± 0.006 mm at M1, $p < 0.05$; 0.140 ± 0.003 *versus* 0.124 ± 0.001 mm at incisor under M1, $p < 0.001$; 0.148 ± 0.007 *versus* 0.112 ± 0.006 mm at M2, $p < 0.01$; and 0.142 ± 0.002 *versus* 0.127 ± 0.002 mm at incisor under M2, $p < 0.01$; respectively). Panel C: nanoindentation analysis of the incisor and the molar on frontal sections of the mandible performed at the level of M1 shows non-significant (NS) difference between KO and WT mice for enamel and dentin hardness and Young's modulus. e: enamel; dfp: dentin far from pulp; dmr: dentin middle region. * indicates a statistically significant difference with the WT.

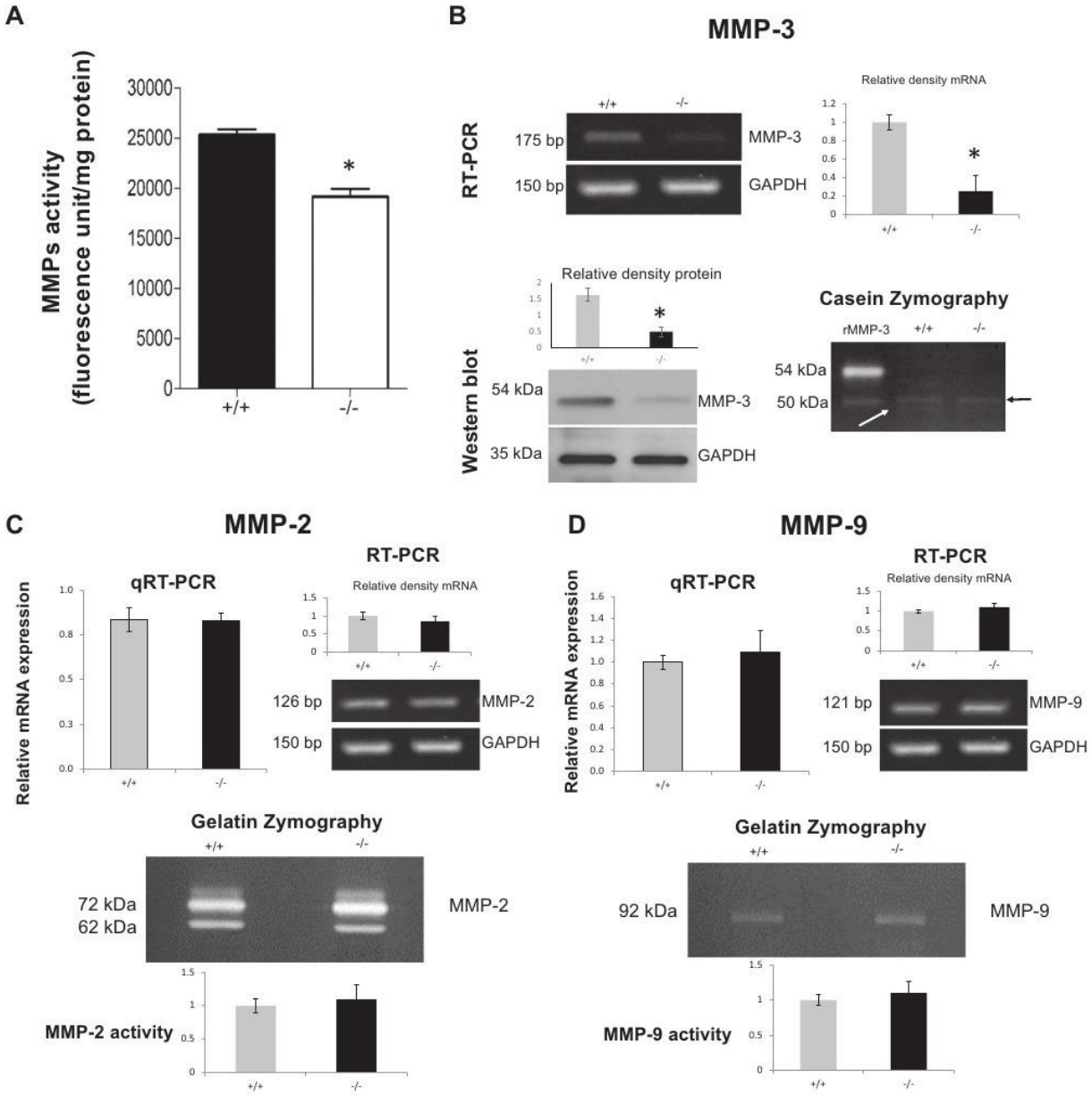

Fig. 3. Biochemical MMP evaluation of molar germs and developing incisor from EMMPRIN KO mice when compared with WT. Panel A: extracts from molar germs (M1 + M2) collected from WT and KO post-natal day 5 mice were analyzed for total MMP activity. A significant decrease is observed for KO (19,130 ± 769) compared to WT mice (25,300 ± 560). Similar results were found for lower incisor germs (soft part) collected in 3-month-old mice. Panel B: for MMP-3 mRNA expression, a 75% decrease is observed for KO mice when compared with WT by RT-PCR analysis of developing incisor extracts. For protein expression, a band is detected at about 50 kDa in both groups but with a 66% decrease in KO mice by Western blot analysis. MMP-3 activity is observed by casein zymography as a band at 50 kDa (black arrow) in both KO and WT groups, but the band appears weaker in KO. A fainter band is also detectable underneath the major band in WT (white arrow), but not in KO mice. Recombinant MMP-3 is observed at 54 and 50 kDa. Panels C: for MMP-2 mRNA expression in developing incisor extracts, no detectable difference is observed for KO mice when compared with WT by qRT-PCR. Zymographic analysis shows no difference in MMP-2 activity between WT and KO. For both, 2 bands around 72 kDa corresponding to proMMP-2 and a band at 62 kDa corresponding to the active MMP-2 are observed. Panel D: for MMP-9 mRNA expression, no difference is observed for KO mice when compared with WT by qRT-PCR. Zymographic analysis shows no difference in MMP-9 activity between WT and KO mice. For both, a band at 92 kDa corresponding to pro-MMP-9 is detected. * indicates a statistically significant difference with the WT group.

Lower MMP activity in the tooth germs of EMMPRIN deficient mice

Molar and incisor germ extracts were analyzed for total MMP activity using a broad ranged fluorescent substrate for MMPs (Fig. 3A). A decrease of 25% in activity was observed in the extracts of EMMPRIN KO mice when compared with WT mice. MMP-3 expression was shown to be about 70% lower in KO mice by both RT-PCR and immunoblot (Fig. 3B). Lower MMP-3 activity in KO was detected by casein zymography (Fig. 3B). In contrast, analysis of the transcript and protein levels of MMP-2 and MMP-9 showed no detectable difference between WT and KO animals (Figs. 3C–D). MMP-20 expression was significantly lower in KO mice at both the transcript and protein levels (Fig. 4A),

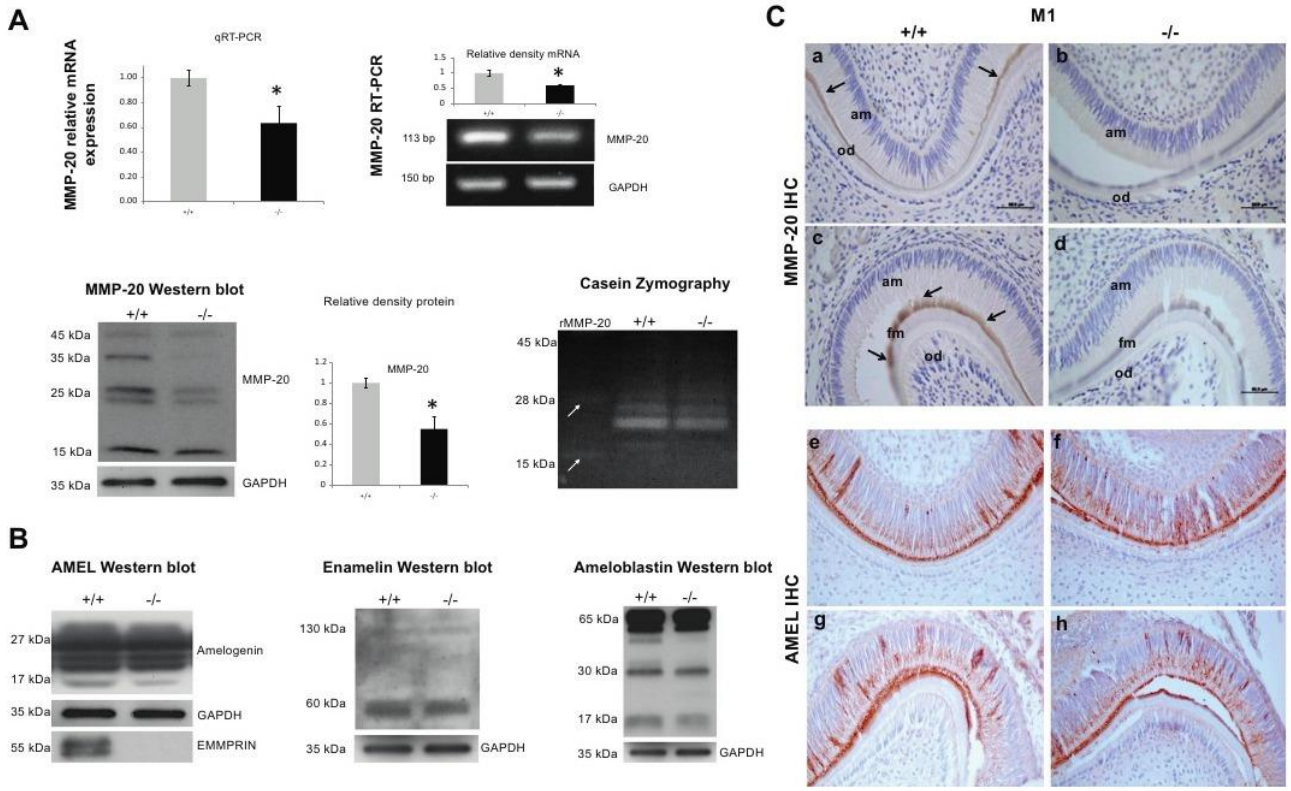

Fig. 4. MMP-20, and enamel matrix protein expression in tooth germs of EMMPRIN KO mice when compared with WT. Panel A: for MMP-20 mRNA expression, a 35% decrease is observed for KO when compared with WT by RT-PCR analysis. For protein expression, several bands are detected with MMP-20 antibody between 45 kDa and 15 kDa in both samples but with about 50% decrease in KO mice. MMP-20 activity is observed by casein zymography as several bands detectable between 45 and 15 kDa in both KO and WT groups, the strongest digestion band being observed at about 25 kDa. All the bands appear weaker in KO. Recombinant MMP-20 activity is observed at 28 and 18 kDa (white arrows). Panel B: Western blot analysis shows with amelogenin (AMEL) antibody several bands extending from 27 to 17 kDa for WT and KO mice, with enamelin antibody a small band at 130 kDa and a larger band at about 60 kDa, and with ameloblastin antibody several bands extending from 65 to 17 kDa. No significant difference is observed between groups, but for ameloblastin some faint intermediate bands seen in WT mice are hardly detectable in KO. Western blot analysis with EMMPRIN antibody confirmed the absence of EMMPRIN in KO. Panel C: For MMP-20 expression, a staining of the matrix associated with the secreting pole of ameloblasts (am) is observed in WT (a, c). It increases progressively from the intercuspal curve (a) to the tip of the cusps (c) where forming dentin (fm) is seen. A faint staining is detected in KO mice (b–d) at the apical pole of ameloblasts located at the tip of the cusps (d). For AMEL expression, the staining is associated with secreting ameloblasts (am) and is particularly intense at the secretory pole. No difference is seen between WT (e, g) and KO (f, h) germs. am: ameloblast, od: odontoblast; fm: forming matrix. * indicates a statistically significant difference with the WT group.

and fainter MMP-20 activity was observed by casein zymography (Fig. 4A). The decrease in MMP-20 expression was confirmed by immunostaining in the M1 germ (Fig. 4C, a–d). In WT mice, MMP-20 staining was associated with the matrix at the interface with the secreting pole of ameloblasts. Staining increased progressively from the less differentiated cells located in the intercuspal curve (Figs. 4C, a), reaching maximal intensity at the tip of the cusps where the cells are fully differentiated (Figs. 4C, c). By contrast, in KO mice, only weak staining could be discerned in the neo-formed matrix at the tip of the cusps. We then sought to determine whether the decrease in MMP-20 was associated with a modification in enamel matrix protein cleavage. AMEL immunostaining in the M1 germ was localized mainly at the secreting pole of the ameloblasts, particularly intense at the tip of the cusps which corresponds to the start of enamel synthesis but there was no notable difference between WT and KO mice (Figs. 4C, e–h). In addition, a normal profile of the protein expression was shown in the Western blot analysis of tooth germ extracts (Fig. 4B). Similar results were obtained by Western blot analysis for the expression of enamelin (Fig. 4B). Regarding ameloblastin expression, several bands were detected from 65 to 17 kDa, some of the intermediate bands being slightly less detectable in KO. The analysis of the transcripts of Kallicrein-4 (KLK-4), an enzyme involved in late enamel maturation [16], showed a significantly higher level of expression in KO mice, which however could not be detected at the protein level by either western blot (data not shown) or casein zymographic analysis (Supplementary Fig. 2).

EMMPRIN regulates the expression of several MMPs in tooth germ cultures

To further assess the direct role of EMMPRIN on MMP expression, we investigated the effects of EMMPRIN inhibition on tooth germs in culture [48]. In that purpose, molar germs sampled at E18 were cultured on semi-solid culture medium for 1 day prior to inhibition by siRNA. The germs were then treated for 48 h with two different concentrations of EMMPRIN siRNA (Fig. 5). RT-PCR analysis showed that EMMPRIN expression was notably diminished at both siRNA concentrations (63% and 90%, respectively). This decrease had no detectable effect on the expression of MMP-2, while a significant decrease of MMP-3 (50% and 65%, respectively) and MMP-20 (about 80% for both siRNA concentrations) expression was observed. In addition, a 20% decrease was also observed with the highest siRNA concentration for MMP-9.

Delayed basement membrane degradation in the molar germs of EMMPRIN deficient mice

As the expression of MMP-3 is lowered in EMMPRIN KO mice and when EMMPRIN is silenced by siRNA, we explored the degradation of

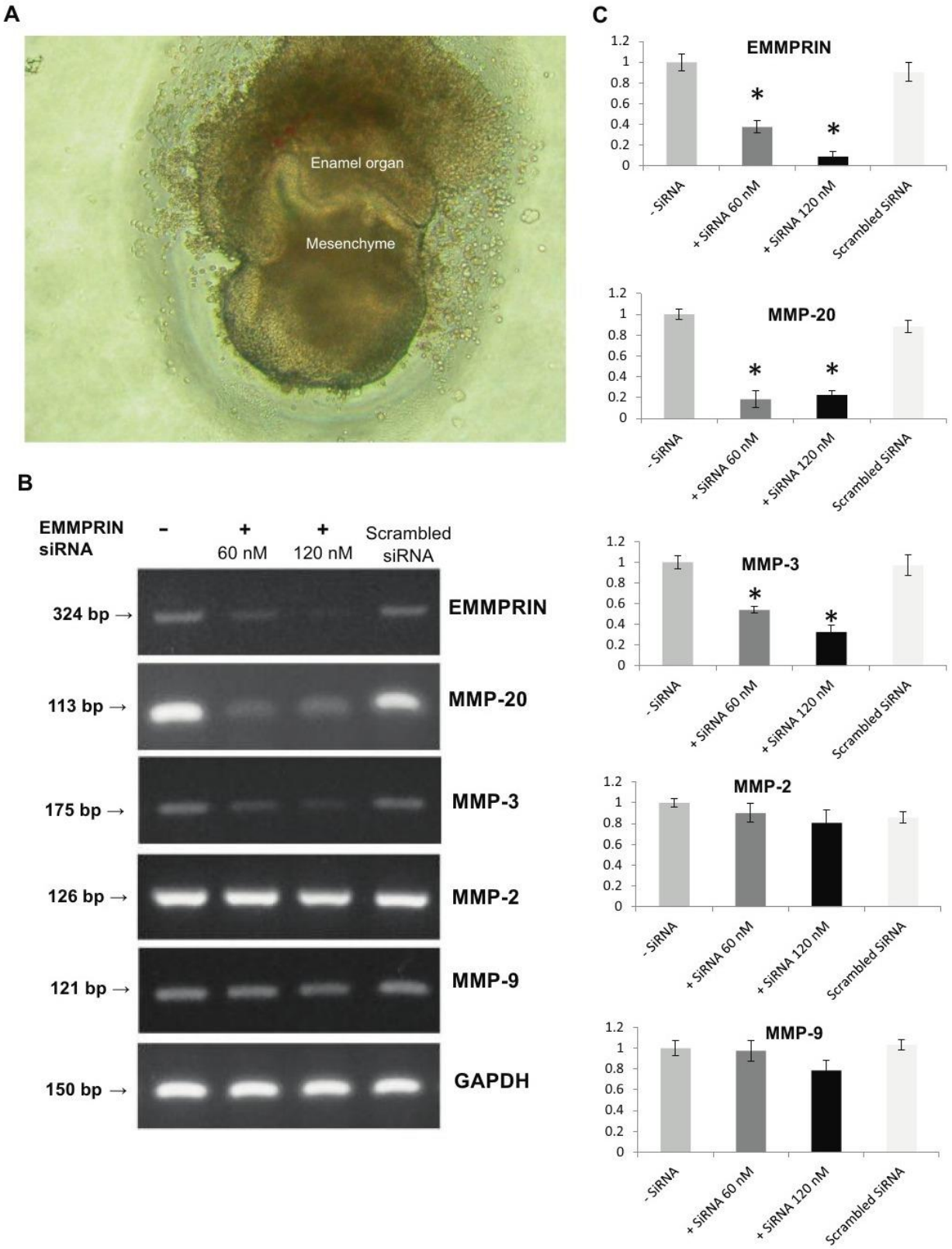

Fig. 5. Treatment of tooth germs with EMMPRIN SiRNA. A: mouse M1 germ sampled at E18 and cultured for 24 h on semi-solid medium. B: RT-PCR analysis of total RNA extraction from cultured M1 germs treated for 48 h with EMMPRIN siRNA (lane 1: no siRNA; lane 2: siRNA 60 nM; lane 3: siRNA 120 nM; lane 4: scrambled siRNA). C: RT-PCR quantification with image J software showing that EMMPRIN expression is decreased at both siRNA concentrations (63% and 90%, respectively) leading to a significant decrease in MMP-20 (about 80% for both siRNA concentrations), and MMP-3 (50% and 65%, respectively) expression. No significant decrease is observed for MMP-2 and MMP-9. * indicates a statistically significant difference with the "no siRNA" treatment group.

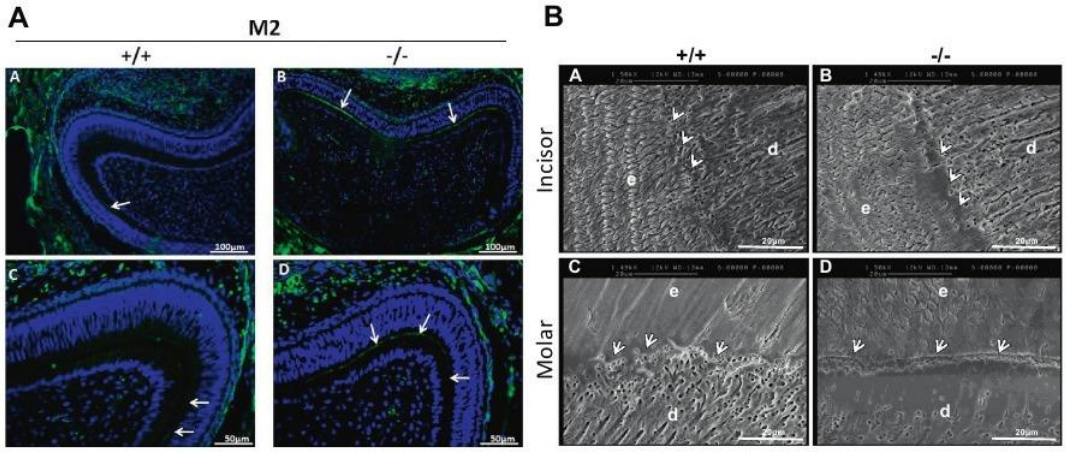

Fig. 6. Dentino-enamel junction observation of EMMPRIN KO and WT mice. Panel A: laminin fluorescent staining in M2 germ from EMMPRIN KO and WT mice. Laminin was analyzed by immunocytochemistry on M2 germs of new born mice to investigate basement membrane degradation. In WT mice, the staining at the level of basement membrane is faint and discontinuous (A, C), whereas in KO it is continuous (white arrows) (B, D). The staining is visible in the intercuspal zone (B) and at the tip of the cusps (D). Panel B: SEM observation of 3 month-old mouse mandible sections. In control, the DEJ (white arrows) is formed by well-intricate tissues in M1 (C) and in the underlying developing incisor (A). In contrast, these interactions between dentin and enamel are not visible in the EMMPRIN KO mice and a thick line separates both compartments (B, D). This layer is observed as well in the molar (D) as in the developing underlying incisor (B). e: enamel. d: dentin.

the basement membrane at early bell stage by fluorescent immunolabeling of laminin, one of its major components (Fig. 6A). Laminin immunostaining appeared continuous in EMMPRIN KO mice while it was faint and discontinuous in the WT, suggesting delayed basement degradation in the absence of EMMPRIN. In order to assess the consequences of this delayed degradation in the adult mice, scanning electron microscopy (SEM) analysis of sections of 3 month-old mandibles was performed at the level of M1. Results revealed that whereas both dentin and enamel appeared to retain their normal structure in EMMPRIN KO mice (Supplementary Fig. 3), clear differences could be discerned at the dentino-enamel junction (DEJ), the boundary between these two anatomical structures, in molars (Figs. 6B, C–D) and developing incisor (Figs. 6B, A–B). While the DEJ in WT mice appeared as a well-intricate structure providing a solid anchorage of enamel within dentin, it consisted of a rough calcified layer of approximately 10 ± 5 µm (mean ± SD) in the KO mice.

Discussion

EMMPRIN/CD147 is a MMP inducer considered as an important mediator of epithelio–stromal interactions in several normal and pathological processes [22]. EMMPRIN is known to be expressed during rodent tooth formation [37,38], and data obtained from *ex-vivo* germ culture have suggested that it was involved in tooth morphogenesis [38]. However, the precise *in vivo* function of this molecule in dental tissues remained to be elucidated. This study shows that EMMPRIN KO adult mice, although globally displaying a normal tooth morphology, present several dental alterations including decreased enamel volume and thickness, likely resulting from a downstream action of EMMPRIN on MMPs. Indeed, we showed that the expression and activity of MMP-3, which is able to degrade basement membrane components [49], and MMP-20 that is necessary for enamel formation [16], are down-regulated in teeth of EMMPRIN KO mice, as well as upon EMMPRIN siRNA inhibition.

The proteolytic disruption of the basement membrane during tooth development is a key step that promotes tissue remodeling through the modification of the epithelial-mesenchymal interactions [8]. This disintegration allows a direct interaction between these two tissues necessary for terminal cell differentiation. Here we demonstrate that EMMPRIN, *via* regulation of MMP expression, is involved in the disruption of the anatomical barrier that separates dental epithelium from mesenchyme. The direct cell-to-cell contact allowed by basement membrane degradation was delayed in the teeth of EMMPRIN KO mice, as suggested by the persisting laminin immunostaining. This is consistent with the inhibition of MMP-3 which is known to be involved in the basement membrane degradation [49]. Consequently, an enamel mineralization delay was observed in the growing incisors of all the explored EMMPRIN KO mice. Although technically difficult to confirm, it is expected that molar germs also display enamel mineralization delay. This assumption is strengthened by the delay observed in basement membrane degradation in the molar germs, and by the defects observed at the DEJ in both adult molars and incisors.

Our results show that EMMPRIN is intensely expressed in epithelial cells (*i.e.* pre-ameloblasts and stratum intermedium) of developing teeth prior to dentin and enamel formation. These data confirm previous findings showing the increasing expression of EMMPRIN from the bud to the bell stage in stratum intermedium and inner dental epithelium layers [37,38]. However, at more advanced developmental stages (start of dentin and enamel deposition), we demonstrate that EMMPRIN protein is also expressed by odontoblasts. This rapid switch suggests an involvement of EMMPRIN in the well-tuned epithelial–mesenchymal dialogue that allows tooth development.

MMP-20 production was also decreased in dental tissues of EMMPRIN KO mice. MMP-20, which is a tooth-specific enzyme [18], is implicated in the early stages of enamel maturation and proposed to be solely responsible for AMEL processing during the secretory stage [17]. At this early stage of enamel maturation, MMP-20 has also been shown to be responsible of the cleavage of enamelin and ameloblastin, two other enamel matrix proteins [39,40]. However, the decreased MMP-20 expression observed in the teeth of the EMMPRIN KO mice were solely associated with decreased enamel volume and thickness, which did not match the severe enamel abnormalities observed in the teeth of the MMP-20 KO mice [50–52]. In addition, the nanoindentation study did not reveal any differences between EMMPRIN KO and WT mice, suggesting a rather normal enamel mineralization. This contrasts the tooth phenotype observed in the MMP-20 KO mice [53]. Indeed, MMP-20 KO mice exhibit a severe tooth phenotype with altered rod pattern and hypoplastic enamel that delaminates from the dentin [51,54]. This abnormal phenotype was suggested to result from the lack of MMP-20-dependent AMEL cleavage, highlighting the importance of enamel matrix proteolysis for proper enamel formation. Normal enamel mineralization in teeth of EMMPRIN KO mice may be

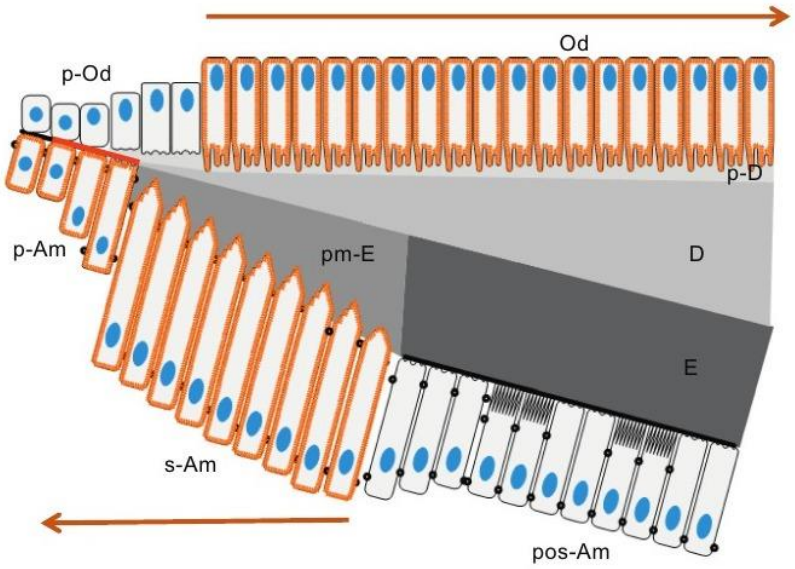

Fig. 7. Recapitulative schema proposing the role of EMMPRIN in tooth formation. At early bell stage, EMMPRIN is expressed by pre-ameloblast (p-Am) and may orchestrate basement membrane degradation (black line) to allow direct contact with pre-odontoblast (p-Od), which is mandatory for the final cell differentiation. At secretory stage, both secreting ameloblasts (s-Am) and odontoblasts (Od) highly express EMMPRIN. This expression may enhance MMP-20 synthesis by ameloblasts allowing for early enamel maturation. At the enamel maturation stage, post-secretory ameloblasts (pos-Am) lose their EMMPRIN expression. The arrows indicate EMMPRIN expression by cells. The red line schematizes the time window where a direct effect of EMMPRIN is allowed by a direct cell contact. D: dentin; pD: predentin; pm-E: premature enamel; E: enamel. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

explained by the fact that MMP-20, although markedly decreased, may still be sufficient to cleave enamel matrix proteins during early enamel maturation.

Interestingly, expression and activity of MMP-2 and MMP-9, which are regulated by EMMPRIN in several tissues and various pathological and repair conditions such as tumor invasion and corneal wound healing [23,28,32], remained normal in dental tissues of EMMPRIN KO mice. This highlights a complex mechanism of MMP regulation by EMMPRIN, which depends on the tissue context. Altogether, the mild consequences of the absence of EMMPRIN on the adult tooth phenotype, in spite of the lower expression of both MMP-3 and MMP-20, may indicate that the level of MMP expressed in the developing tooth germs in EMMPRIN KO mice is sufficient to allow for a correct tooth development, although the existence of a compensatory mechanism implicating other proteases cannot be excluded. In addition, the direct effect of EMMPRIN on the epithelial–stromal interaction may be limited since it is only allowed during a short time window, namely between basement membrane degradation allowing direct cell contact and before calcified matrix deposition which constitutes cell barriers, hence limiting EMMPRIN's scope of action (Fig. 7).

In conclusion, our study suggests that EMMPRIN, through the induction of proteases, may orchestrate the epithelial–mesenchymal cross-talk necessary for tooth formation, by enabling cleavage of the basement membrane and thus direct cell–cell interactions. At more advanced stages, EMMPRIN can also facilitate enamel maturation by inducing proteases such as MMP-20 that are capable of cleaving matrix components.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bone.2014.06.019.

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All authors state that they have no conflicts of interest.

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3.1.1 Supplementary data

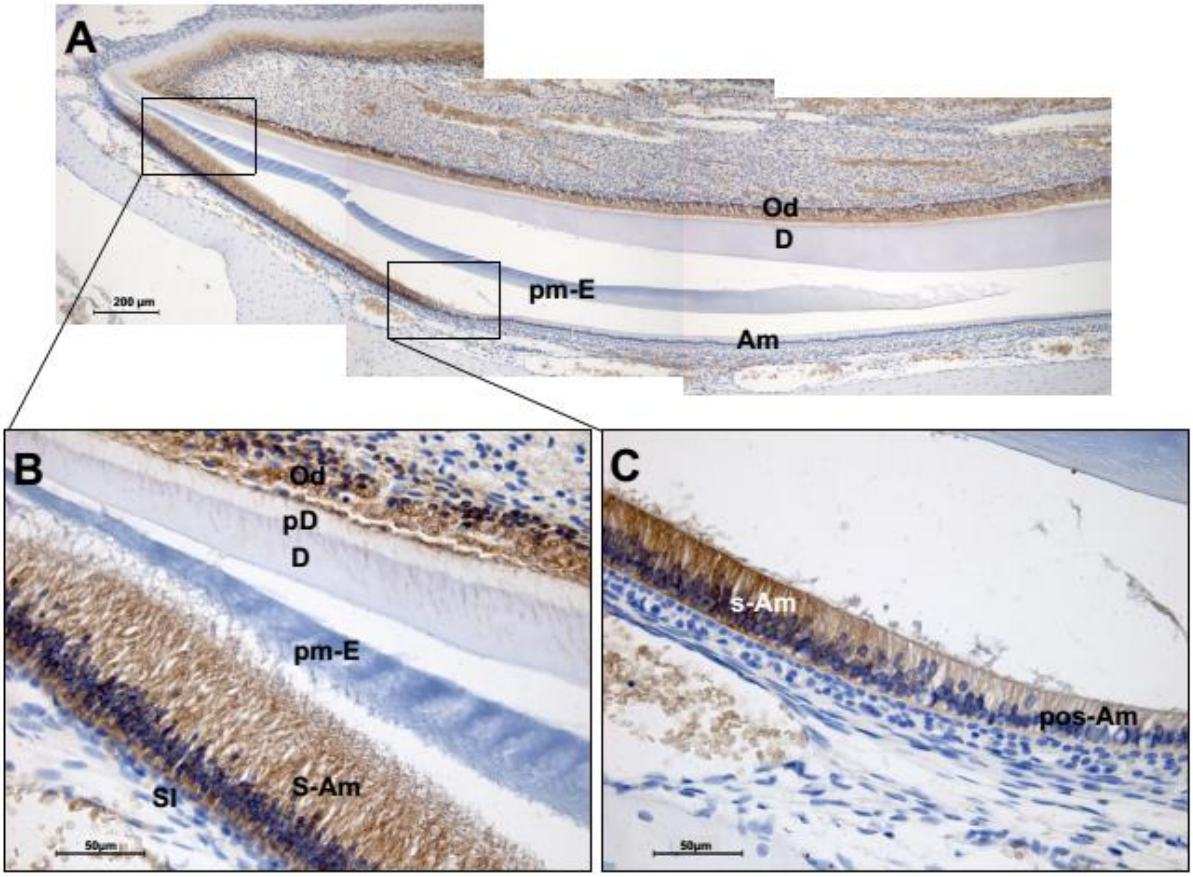
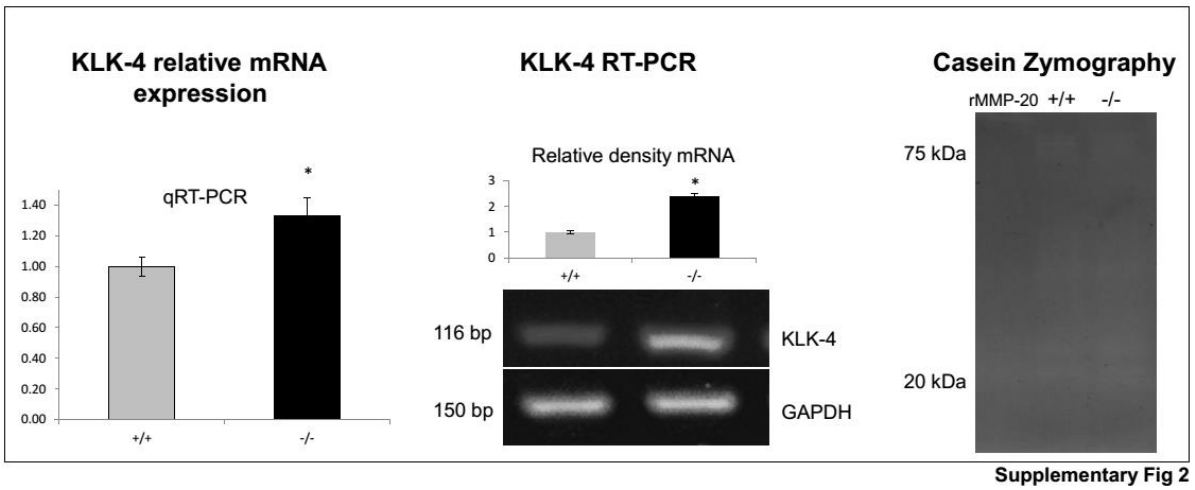


Figure 22 : **EMMPRIN expression in the developing incisor of 3 month-old mice Immunostaining with EMMPRIN antibody on sagittal section of the mandible shows that the secretory ameloblasts, the stratum intermedium and odontoblasts are positive for EMMPRIN (A and B). By contrast, no staining is observed in the post-secretory ameloblast (C). Am: ameloblast; s-Am: secretory ameloblast; pos-Am: post-secretory ameloblast; Od: odontoblast; D: dentin; pD: predentin; pm-E: premature enamel; Si: stratum intermedium; fm: forming matrix. From** *(Khaddam et al. 2014)*



Supplementary Fig 2

Figure 23 : **KLK-4 expression in tooth germs of EMMPRIN KO mice when compared with WT. For mRNA expression, a 33 % increase is observed by qRT-PCR in KO mice. KLK-4 activity is hardly detectable by casein zymography (with 20 mM EDTA in the incubation buffer to inhibit MMP activity). No activity is seen for recombinant MMP-20. From** *(Khaddam et al. 2014)*

Supplementary Table 1 : **PCR gene primers and reference. From** *(Khaddam et al. 2014)***.**

	5' – 3' Forward primer	5' – 3' Reverse primer	Product
EMMPRIN NM_009768.2	CGCTGGCCTTCACGCTCTTGA	TTGCGGTCTGGGTCACTGCT	324 bp
GAPDH NM_008084.2	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG	150 bp
MMP-2 NM_008610.2	GCTGATACTGACACTGGTACTG	GTCCTTGAAGAAGAAGATCTCA	126 bp
MMP-3 NM_010809.1	GCTGAGGACTTTCCAGGTGT	GCCTTCCTTGGATCTCTTTTTA	175 bp
MMP-9 NM_013599.3	GGTGATCTCTTCTAGAGACTGG	CTAAAGTAGCTGGAAAAGGTT	121 bp
MMP-20 NM_013903.2	GGAGTGAGTGGCCATATAGA	ACCACACTAACCACGTCTTC	113 bp
KLK-4 NM_019928.1	AACATGATGGTCACTGCAC	CAGTCCTGGCCTTGTATGAT	116 bp

Supplementary table 1

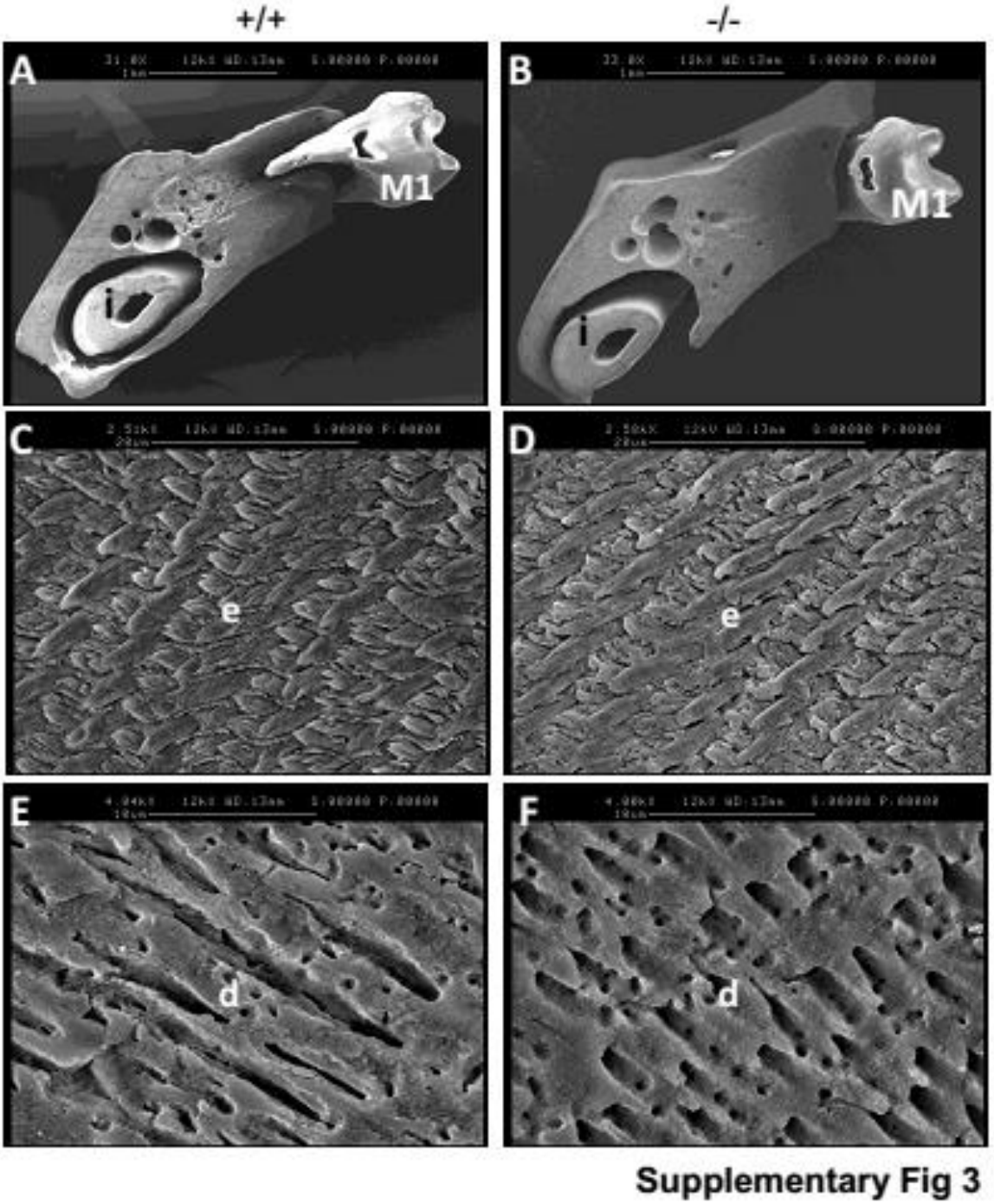


Figure 24 : **SEM observation of 3 month-old mouse mandible sections. At M1 level, no difference in the morphology of either the bone or the teeth is detected between WT and KO mice (A, B). Both dentin (E, F) and enamel appear normal and the enamel prisms are normally constituted (C, D). From** *(Khaddam et al. 2014)***.**

3.1.2 Supplementary results

3.1.2.1 Basement membrane degradation - Transmission electron microscopy

Basement membrane degradation is delayed in the molar germs of EMMPRIN KO when compared to WT mice

3.1.2.1.1 Materials and methods

Mandibles of post-natal day 1 mice (3 litter-mate mice per group) were analyzed by conventional transmission electron microscopy (TEM). Heads were fixed in 2% (w/v) glutaraldehyde in 0.15 M cacodylate buffer, pH 7.4, overnight at 4°C. After post-fixation in 2% OsO4 for 1 h and dehydration in graded ethanol series at 4°C, the samples were embedded in Epon 812 (Fluka). Semi-thin sections were stained with toluidine blue and fuchsine. After washing the sections were dried and mounted in Eukitt. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined with a JEOL 1011 electron microscope.

3.1.2.1.2 Results

To explore EMMPRIN role in mediating direct ameloblast-odontoblast interactions, we performed transmission electron microscopy analysis on the molar germs of new born mice. TEM examination of M1 and M2 germs allowed the observation of the cells located at the tip of the cusps which corresponds to the higher differentiation stage (**Figure 25**). On M2 germs, the cell polarization observed in the WT (**Figure 25**.A) was not visible in the KO in either the ameloblasts and the odontoblast layer (**Figure 25**.B). The basement membrane, which separates the pre-ameloblast from the pre-odontoblast compartments, was already partially degraded in the WT allowing for direct cell interactions between the two cellular compartments (**Figure 25**.C), whereas it was still continuous in the KO (**Figure 25**.D), suggesting that the differentiation process was delayed in EMMPRIN KO germs. However at a later stage of the development (M1 germ), cells were fully polarized with a palisade organization in both mice (**Figure 25**.E, F). The basement membrane at the tip of the cusp was no longer detectable in either mice model (**Figure 25**.G,H). Dentin matrix was actively secreted and mineralized although at a more advanced stage in the WT and mineralizing enamel was already detectable (**Figure 25**.G).

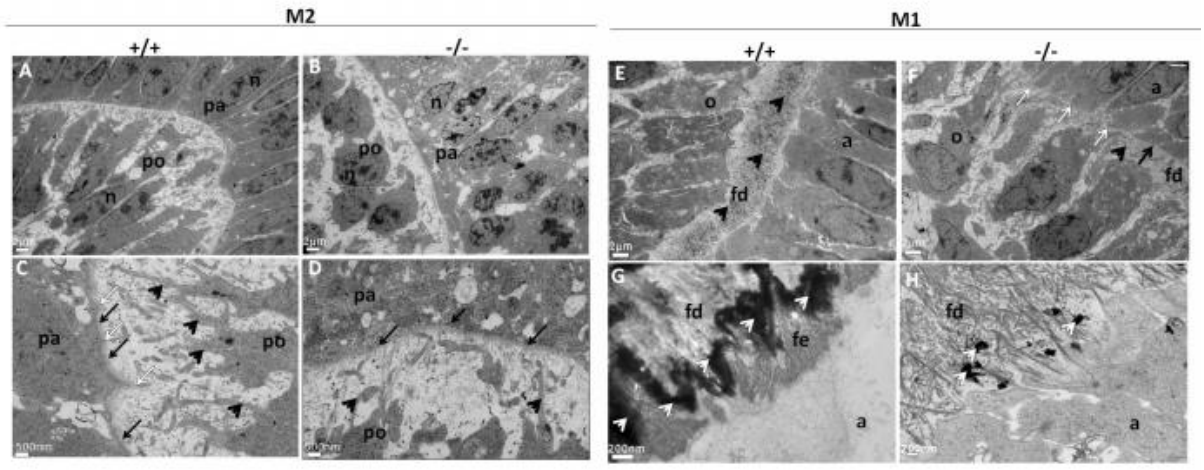


Figure 25: **TEM analysis was performed on M1 and M2 germs of new born mice. In the KO M2 germs, a cell polarization delay is observed in both pre-ameloblasts and pre-odontoblasts localized at the tip of the cusps (b). In the WT, well-organized ameloblast and odontoblast palisades are seen, with a basal localization of the nuclei and long cell processes (arrow-heads) (a), whereas in the KO, cells are seen proliferating with centrally localized nucleus (b). At higher magnification, the basement membrane (black arrows) is partially degraded in WT (white arrows) (c), but appears still intact in the KO (d). In M1 germ, the basement membrane which can no longer be detected in the WT (e) is partially degraded in the KO (arrow) (f). Dentin matrix (black arrow-heads) is secreted in both mice models (e-f-g-h) but at a higher rate in the WT (e) where a greater amount of fibrillated collagen is seen associated with hydroxyapatite crystals (white arrow heads). In addition, mineralizing enamel matrix can already be observed at the secreting pole of WT ameloblasts localized at the tip of the cusp (g) but is not detectable in the KO (h). pam: pre-ameloblast; pod: pre-odontoblast; am: ameloblast; od: odontoblast; fde: forming dentin; fen: forming enamel. (Own data).**

3.1.2.2 EMMPRIN expression in the molar germ of mouse embryo

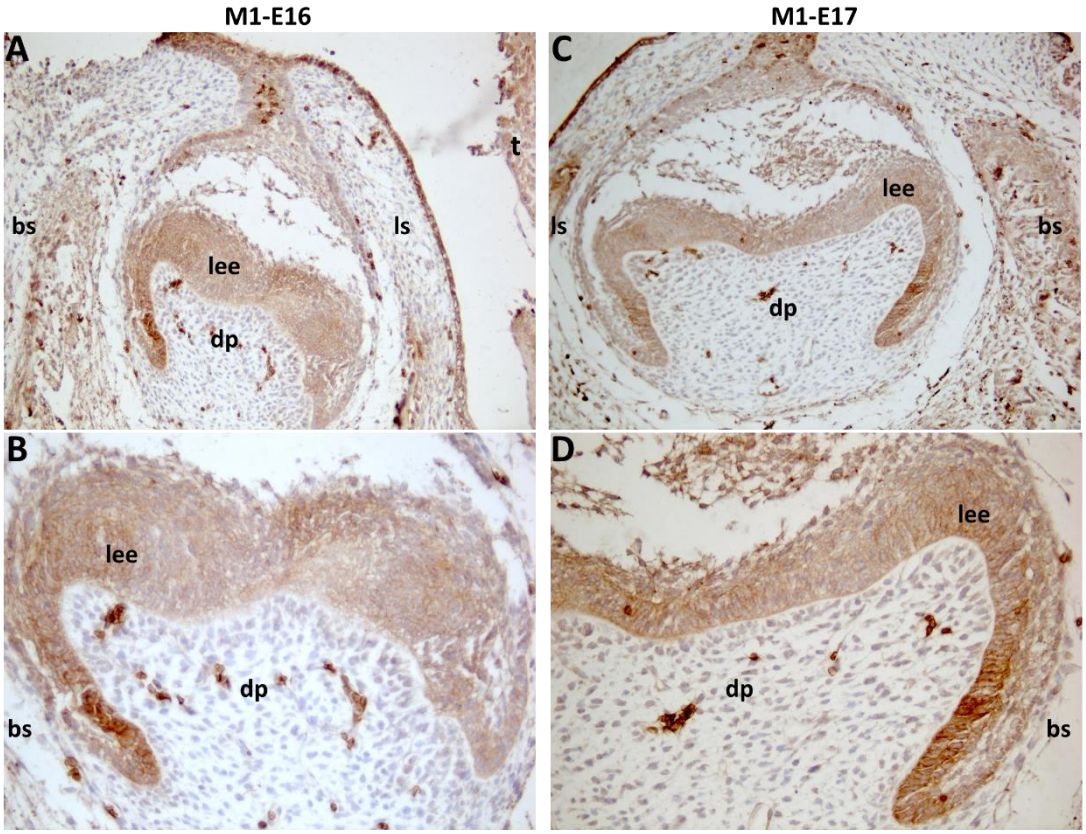


Figure 26: **EMMPRIN expression in the first molar of mouse embryo. Immunoreactivity (IR) for EMMPRIN in paraffin sections of mouse embryo tooth germ tissue at 16 day and 17 day (cap stage). Inner enamel epithelium cells show EMMPRIN IR, this IR in the buccal side is stronger than in the lingual side of the molar germ. Iee: innerenamel epithelium; dp: dental pulp; bs: buccal side; ls: lingual side. (own data)**

3.1.2.3 Alveolar bone phenotype

3.1.2.3.1 Materials and methods

In order to explore the alveolar bone phenotype in EMMPRIN -/- mice, Half mandibles (n=3 age-matched mice per group) were subjected to a desktop micro-CT, (Skyscan 1172, Skyscan, Aartselaar, Belgium). The micro-CT settings were used as follows: 9 μm resolution, voltage 80 kV; current 100 μA; exposure time 400 ms; 180° rotation; rotation step 0.4 degree; frame averaging 4. The scanning time was approximately 4 hours/sample. A total of 1700 native slice frames per sample were reconstructed using NRecon software (Skyscan, Belgium). Tridimensional images were acquired with an isotropic voxel size of 9.92 μm. Full 3D high-resolution raw data are obtained by rotating both the X-ray source and the flat panel detector 360° around the sample.

Bone volume rendering was measured using the open-source OsiriX imaging software (v3.7.1, distributed under LGPL license, Dr A. Rosset, Geneva, Switzerland) from 2D images.

The microarchitecture of alveolar bone of left mandible was studied. The ventral limit of the volume of interest (VOI) was located at the first section containing the mesial root of the first left molar; the dorsal limit was located 100 sections after, at the level of the alveolar ridge and the buccal surface of the bone jaw (**Figure 27**). The VOI was designed by drawing interactively polygons on the 2D sections. Several polygons were needed to be drawn (e.g. on the first section, several at the middle, and on the final section) using the free hand tool with "CT analyzer" software (Skyscan, release 1.13.5.1, Kontich, Belgium). The interpolated VOI comprised only basal/alveolar bone. A simple global thresholding was determined interactively to eliminate background noise and to select bone.

The parameters analyzed were: Bone volume fraction BV/TV (%), trabecular thickness Tb.Th (mm), trabecular number Tb.N (1/mm), and trabecular separation Tb.Sp (mm). Numerical variables were expressed as mean ± standard deviation.

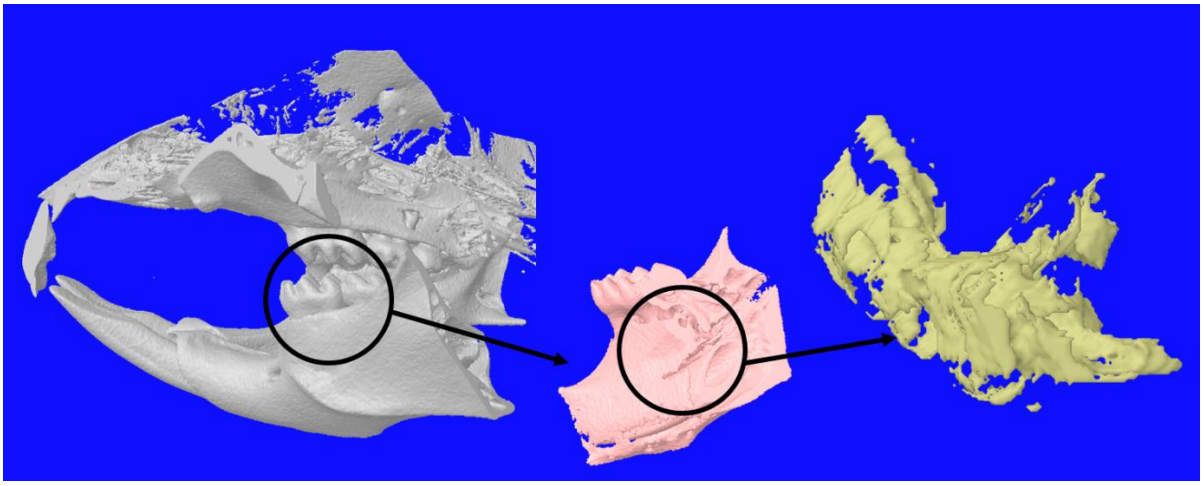


Figure 27: **Alveolar bone density.**

3.1.2.3.2 Results

3.1.2.3.2.1 Bone density

Bone density is presented by the percentage of space occupied by the spongy trabecular bone in the volume of interest (VOI). It was calculated by measuring the ratio between the Percent of trabecular bone volume and bone volume (BV / TV). The BV / TV in +/+ and -/- EMMPRIN mice were almost the same (**Figure 28**).

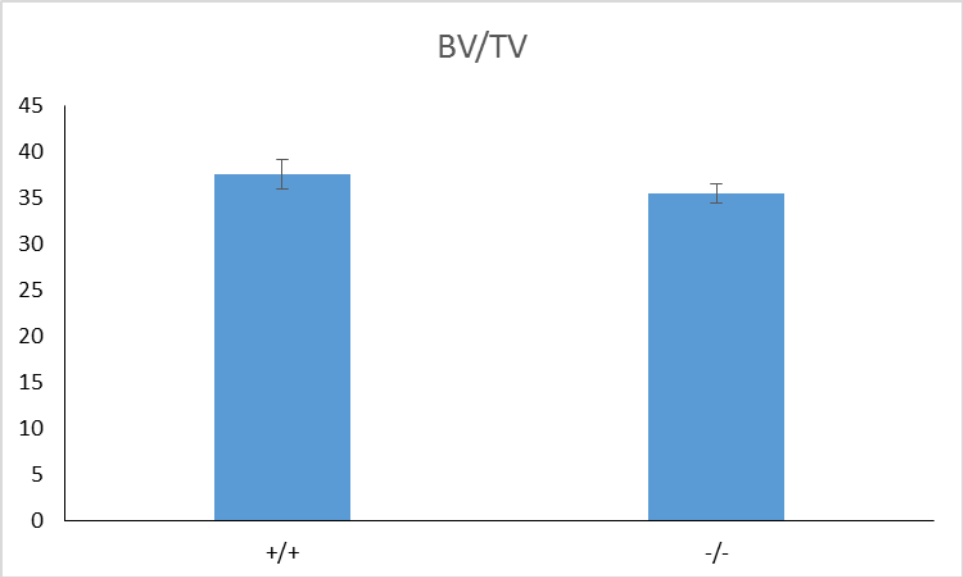
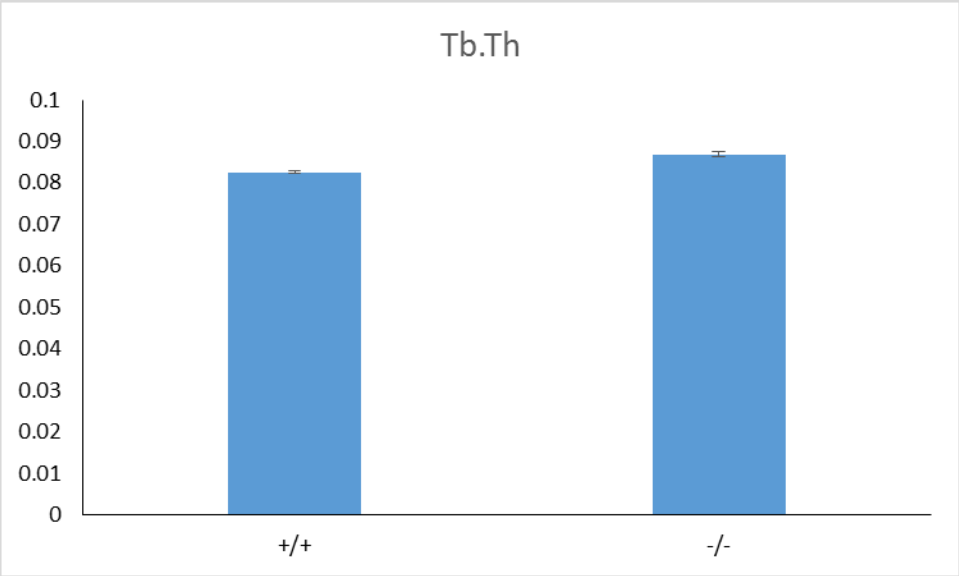


Figure 28: **Percent of bone volume in VOI.**

3.1.2.3.2.2 Trabecular bone characters

Trabecular bone characters are presented by measuring the following:

- Thickness of bone trabeculae (Tb.Th) in the VOI.



The Tb.Th in +/+ and +/- EMMPRIN mice were almost the same (**Figure 29**).

Figure 29: **Trabecular bone thickness in VOI.**

- The number of bone trabeculae (Tb.N) in the VOI.

The Tb.N in -/- EMMPRIN mice were about the same that of +/+ mice (**Figure 30**).

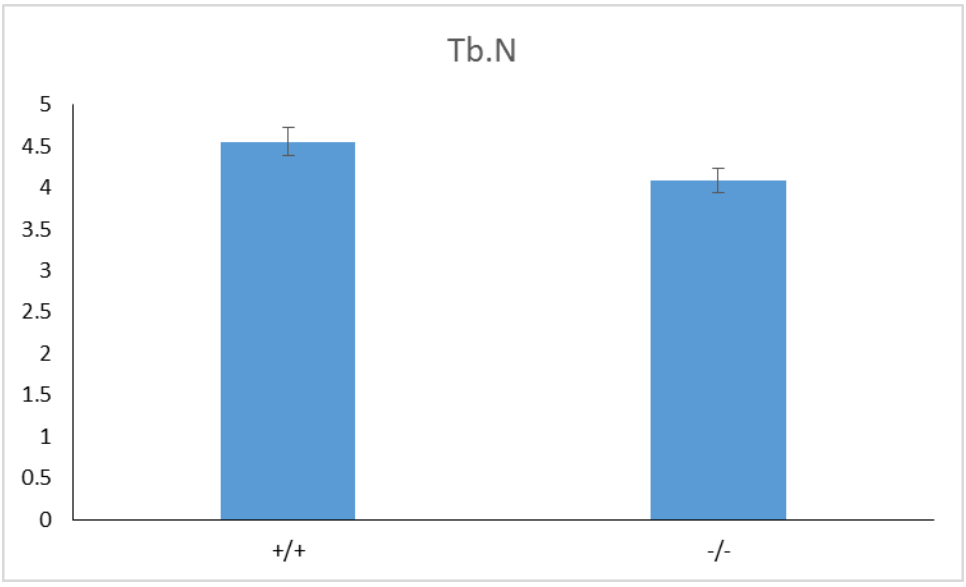


Figure 30: **Trabecular number in VOI.**

- The separation between bone trabeculae (Tb.Sp) in the VOI.

  The Tb.Sp in the -/- EMMPRIN mice were about the same that of +/+ (**Figure 31**).

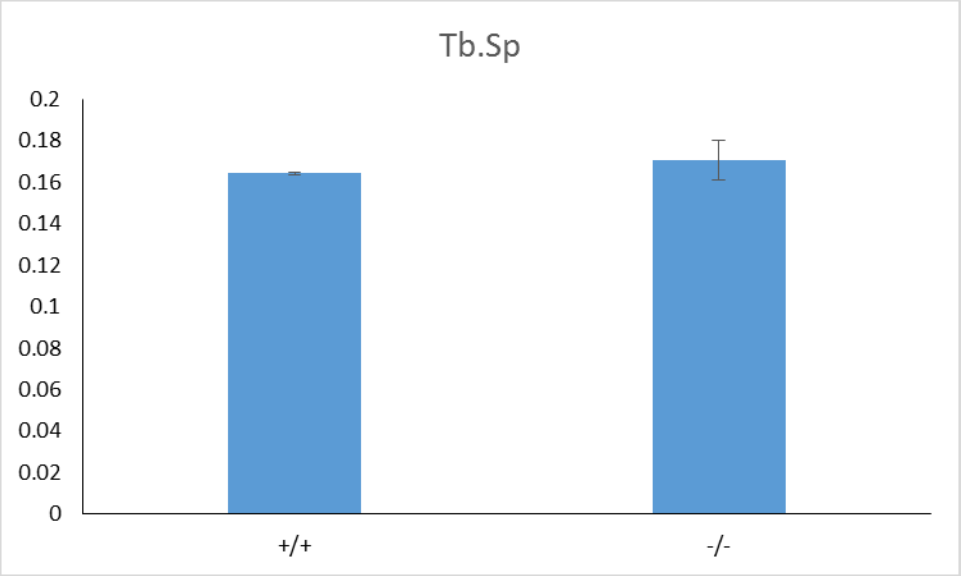


Figure 31: **Trabecular separation in VOI.**

3.2 Role of EMMPRIN in pulp-dentin regeneration

3.2.1 Background and project aim

EMMPRIN has been shown to be involved in the repair process of different injured tissues. Indeed, the role of EMMPRIN in wound healing through MMP induction and increase in myofibroblast contractile activity has been established (Gabison, Mourah, et al. 2005; Huet, Vallée, et al. 2008). Therefore, the aim of this project was to investigate EMMPRIN role in the pulp dentin repair process by comparing the healing of injured pulps of EMMPRIN KO and WT mice.

3.2.2 Materials and methods

Twelve young adult mice (3 month-old) were used (6 WT mice and 6 EMMPRIN KO mice; ethical agreement Animal Care Committee of the University Paris Descartes CEEA34.CC.016.11). Following anaesthesia by intra-peritoneal injection of 2,2,2 tribromoethanol 2-methyl 2-butanol (Avertine®- Sigma Aldrich Germany) (0,017ml/g), a small cavity was prepared with a carbide bur (Dia 0,04mm) (Komet- France) on the acclusal aspect of the first upper left and right molars, in the centre of the tooth according to the mesio-distal plane until the pulp was visible through the transparency of the dentine floor of the cavity. A pulp exposure was mechanically done using an endodontic hand file of 0.15mm diameter with a 4% taper (C+file®, Dentsply-Maillefer France). Pulp capping was performed using Biodentine cement (septodont France) following the manufacturer's recommendations. Using the tip of a probe, Biodentine was placed in contact with the pulp, and slightly condensed with a sterile paper point (XX-Fine, Henry Schein, France). Then, the cavity was sealed with glass ionomer cement (GIC). Animals were placed in individual cages until they recovered from anesthesia, and ibuprofen (0.06mg/g/day) was added in their drinking water for 72 hours. Treated animals were sacrificed at increasing time points following the clinical procedure, as follows:

- Six animals at 1 week post-operatively

- Six animals at 4 weeks post-operatively

Following removal of most of the soft tissues, heads of animals were immersed in 4% para-formaldehyde (PFA) (Sigma) overnight at 4°C. Before demineralization prior to histological analysis, samples were examined by micro-Ct at the same parameters

previously reported (page 75, materials and methods.). Micro-Ct data were analyzed using the Osirix software and then **Ct-analysis software**.

3.2.3 Results

3.2.3.1 Micro-CT

In order to explore the tooth reparation, we performed Micro-CT on the maxilla of the treated WT and KO mice (**Figure 32**).

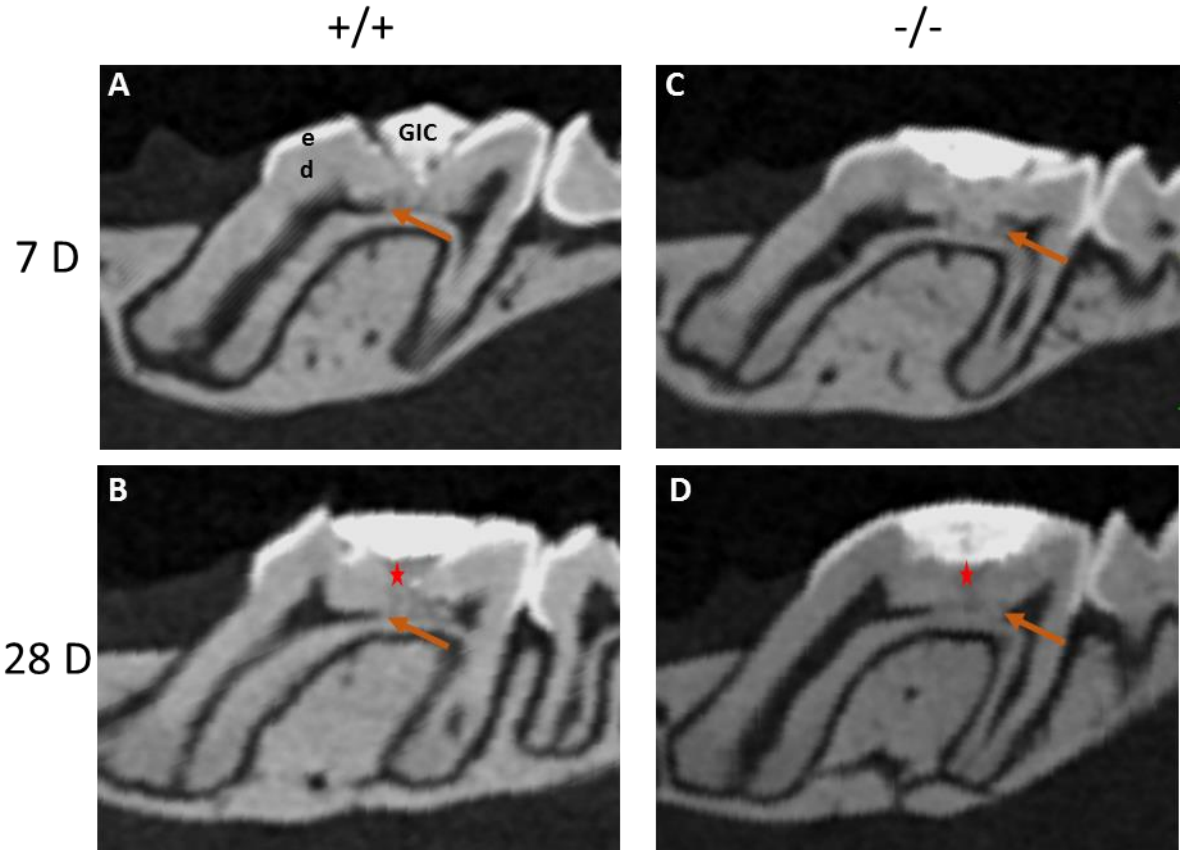


Figure 32: **Mouse first upper molar after 7 and 28 days of capping with Biodentine. 7 days post operatively, dentin formation was detected in +/+ and -/- EMMPRIN mice (A,C), but it was more in -/- (brown arrow C) than in +/+ (brown arrow A). 28 days post operatively, dentine bridge was visible, but it was more continuous in -/- (arrow in D) than in +/+ (arrow in B) where it was not continued. e: enamel; d: dentin; GIC: glass ionomer cement; red *: Biodentine.**

Improved dentin repair is seen in KO when compared with WT both at Day 7 (P= 0.007 S) and day 28 (P= 0.157 NS) (**Figure 33**). This data must now be supported by histological analysis which are ongoing.

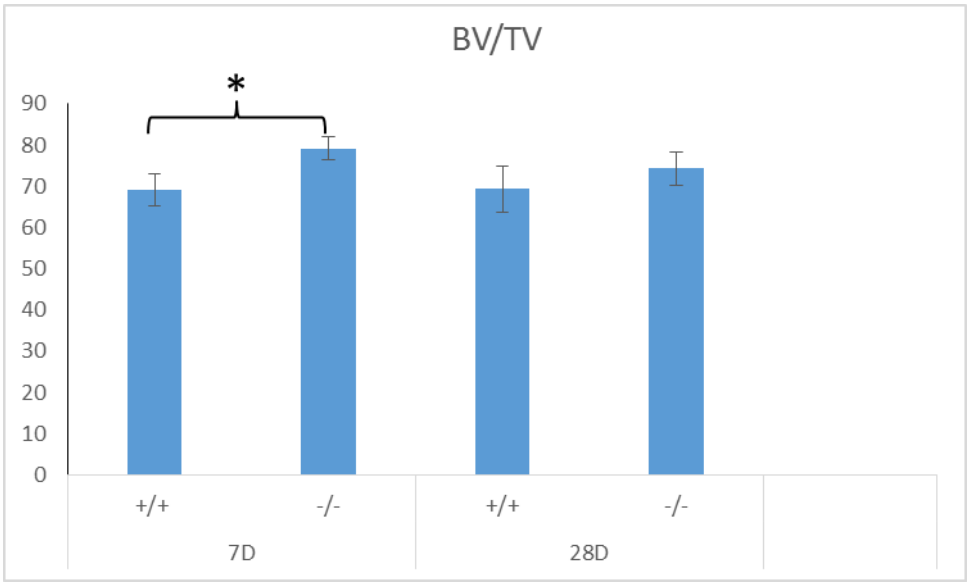


Figure 33: **Percent of dentin volume in volume of interest VOI. Significant increase in dentin density was detected in -/- EMMPRIN mice when compared with +/+ mice at 7 days post operatively.**

3.3 Inhibition of MMP-3 and dentin matrix degradation

frontiers in PHYSIOLOGY

ORIGINAL RESEARCH ARTICLE
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Grape seed extracts inhibit dentin matrix degradation by MMP-3

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Since Matrix metalloproteinases (MMPs) have been suggested to contribute to dentin caries progression, the hypothesis that MMP inhibition would affect the progression of dentin caries is clinically relevant. Grape seed extracts (GSE) have been previously reported to be natural inhibitors of MMPs.

Objective: To evaluate the capacity of a GSE mouthrinse to prevent the degradation of demineralized dentin matrix by MMP-3 (stromelysin-1).

Materials and Methods: Standardized blocks of dentin obtained from sound permanent teeth extracted for orthodontic reasons were demineralized with Ethylenediaminetetraacetic acid (EDTA) and pretreated either with (A) GSE (0.2% w/v), (B) amine fluoride (AmF) (20% w/v), (C) a mouthrinse which contains both, (D) placebo, (E) sodium fluoride (0.15 $mg.ml^{-1}$), (F) PBS, (G) Chlorhexidine digluconate (CHX), or (H) zinc chloride ($ZnCl_2$). The dentin blocks were then incubated with activated recombinant MMP-3. The supernatants were analyzed by Western Blot for several dentin matrix proteins known to be MMP-3 substrate. In parallel, scanning electron microscopy (SEM) was performed on resin replica of the dentin blocks.

Results: Western blot analysis of the supernatants revealed that MMP-3 released from the dentin matrix small proteoglycans (decorin and biglycan) and dentin sialoprotein (DSP) in the AmF, sodium fluoride, PBS and placebo pretreated groups, but not in the GSE and mouthrinse pretreated groups. SEM examination of resin replica showed that the mouthrinse and its active components not only had an anti-MMP action but also modified the dentin surface accessibility.

Conclusion: This study shows that GSE either alone or combined with AmF as in the evaluated mouthrinse limits dentin matrix degradation. This association may be promising to prevent the progression of caries within dentin. However, the procedure should be adapted to clinically relevant durations.

Keywords: grape seed extracts, dentin, matrix metalloproteinases, caries, mouthrinse

INTRODUCTION

Matrix metalloproteinases (MMPs), also designated as matrixins, hydrolyze components of the extracellular matrix (Brinckerhoff and Matrisian, 2002) and play a central role in many biological and pathological processes. Their role in the dentin caries process has also been proposed in several studies (Tjaderhane et al., 1998; Sulkala et al., 2001). MMPs were shown to be expressed during tooth development and to be necessary for normal dentin formation (Bourd-Boittin et al., 2005). After dentin mineralization, they remain trapped in the calcified matrix either under active or proenzyme forms, which may explain their persistent presence within the dentin of adult teeth (Tjaderhane et al., 1998; Mazzoni et al., 2007). It can be therefore hypothesized that their exposure and activation during the caries process would allow these enzymes to promote matrix degradation and caries progression. Indeed, cariogenic bacteria create acidic environment, exposing the dentin matrix. However, cariogenic bacteria cannot degrade dentin matrix after demineralization (Katz et al., 1987), and the bacteria collected from dentinal lesions created *in situ* were unable to degrade collagen *in vitro* (van Strijp et al., 1997). Moreover, purified bacterial collagenases have low degrading activity in acidic environment (Clarkson et al., 1987; Kawasaki and Featherstone, 1997). It can be therefore assumed that the cariogenic demineralization process initiated by the bacteria re-exposes endogenous MMPs and also potentially induces their activation through the acidity they create (Tjaderhane et al., 1998; Sulkala et al., 2001), hence enabling further dentin matrix degradation. In addition, saliva, which bathes the dentin carious lesion,

contains several MMPs that can also participate in the organic matrix degradation (Tjaderhane et al., 1998; van Strijp et al., 2003; Nascimento et al., 2011).

Intra-oral administration of modified tetracylines and zoledronate, known as chemical MMP inhibitors, delayed caries progression in rats (Tjaderhane et al., 1999; Sulkala et al., 2001). These studies suggest that MMPs have a deleterious effect in dentin caries and that MMP inhibition may represent a new avenue for the prevention or treatment of caries. Along this line, several synthetic MMP inhibitors are already in usage in the dental practice. They mainly include Ethylenediaminetetraacetic acid (EDTA) (Thompson et al., 2012), Chlorhexidine digluconate (CHX) (Gendron et al., 1999; Carrilho et al., 2007), or Zinc chloride ($ZnCl_2$) (Toledano et al., 2012) whose MMP inhibitory effect is based on their zinc and calcium chelator properties (Chaussain et al., 2013). In addition, several natural molecules have been previously reported to have MMP inhibitory properties. Grapeseed extracts (GSE) were shown to suppress lipopolysaccharide-induced MMP-1 and MMP-9 secretion by macrophages in culture and to inhibit their activity, and were therefore recommended for use in the development of new treatments of MMP-mediated disorders such as periodontitis (La et al., 2009). Hence, the MMP-inhibitory effects of these natural substances and the fact that they are expected to cause only minimal side effects make them particularly attractive for the treatment of dentin caries, since they can be integrated into the daily-used topical products, such as toothpastes and mouthrinses, or into products used for direct application, such as varnish. In addition, increasing concentrations of GSE have been shown to decrease the degradation rate of the dentin matrix after bacterial collagenase treatment, indicating an inverse relationship between concentration of GSE and collagen solubilization (Bedran-Russo et al., 2008, 2011). Noteworthy, GSE induced collagen inter-microfibrilar cross-links at high concentration (6.5% w/v), modifying the tensile strength properties of the dentin matrix (Bedran-Russo et al., 2011).

Among the MMPs detected in dentin, Stromelysin-1, also termed MMP-3 or proteoglycanase, is capable of degrading the protein core of proteoglycans (PGs), resulting in the release of soluble glycosaaminoglycans (GAGs) (Hall et al., 1999). Boukpessi et al. have previously developed a simplified but well-controlled *in vitro* model to study the dentin caries process, consisting of the pretreatment of human sound dentin cubes by acidic or EDTA solutions followed by a MMP (MMP-3, MMP-2, or MMP-9) treatment of the demineralized matrix (Boukpessi et al., 2008). Using this model, they have shown that MMP-3 released from the collagen scaffold several non-collagenous proteins (NCPs) such as two Small Leucine-Rich Proteoglycans (SLRPs) decorin (DCN), and biglycan (BGN) as well as four members of the SIBLING (Small Integrin-Binding LIgant N-Linked Glycoproteins) family: dentin sialoprotein (DSP), matrix extracellular phosphoglycoprotein (MEPE), bone sialoprotein (BSP), and osteopontin (OPN) (Boukpessi et al., 2008). All these NCPs are known to be associated to the collagen fibers in the dentin (Orsini et al., 2007, 2008) and to participate in the initiation or control of dentin mineralization (Orsini et al., 2012).

Recently, a new daily mouthrinse composed of GSE and amine fluoride (AmF) has been developed. This combination showed both a significant antiplaque activity and an important antioxidant capacity *in vitro*, without any bactericidal effects (Furiga et al., 2014). The objective of this study was to evaluate the capacity of this mouthrinse to prevent dentin matrix degradation by MMPs. As GSE are known to be natural inhibitors of MMPs, we hypothesized that the tested mouthrinse has the ability to limit dentin matrix degradation following MMP treatment, mainly by reducing NCP release. In this study, MMP-3 was selected because we previously showed that it was the only MMP among those tested able to release several NCPs from the dentin matrix (Boukpessi et al., 2008).

MATERIALS AND METHODS

SAMPLES SELECTION

Sound permanent premolars extracted for orthodontic reasons were collected, gently cleaned with tap water and kept at −20°C. All teeth were collected with informed and oral consent from the patients and the parents according to ethical guidelines set by the French law (Loi Bioéthique n°2004-800) and with a special authorization for our team (n°DC-2009-927, Cellule Bioéthique DGRI/A5, Paris, France).

DENTIN DEMINERALIZATION

Enamel was removed by drilling with a high-speed diamond bur under water cooling. Standardized dentin blocks (1 mm^3, 1.2 mg) were prepared from the outer part of teeth with a diamond disk saw under continuous water spray (Accutom 5, Streuers, Copenhagen, Denmark). Blocks were immersed individually at 4°C in 170 µl of 4.13% w/v EDTA (disodic salt) pH 7.2 for 7 days. The demineralizing solution was supplemented with 1/100 Proteinase Inhibitor Cocktail Set V EDTA free (Calbiochem, La Jolla, CA). After demineralization, EDTA was removed by rinsing the blocks three times in PBS.

PRETREATMENT OF DENTIN BLOCKS WITH DIFFERENT SOLUTIONS

Dentin blocks were incubated 48 h at 4°C in different solutions according to the treatment group. The mouthrinse EludrilDAILY® (Pierre Fabre Santé, Castres, France), containing 2 $mg.ml^{-1}$ of GSE (0.2% w/v, Vitimed, Vallon Pont d'Arc, France) and 10.2 $mg.ml^{-1}$ of Fluorinol®, was used as pretreatment solution for group A. The active principles of the mouthrinse were tested separately at the same final concentration found in the mouthrinse: GSE solution (2 $mg.ml^{-1}$, Pierre Fabre Santé) for group B, and AmF (Fluorinol®, 20% w/v; Pierre Fabre Santé) for group C. Placebo, which has the same composition that the mouthrinse without the active principles (GSE and Fluorinol,) was used for group D, sodium fluoride (NaF, N°B48-1450-038 0.15 $mg.ml^{-1}$) for group E, PBS for group F, dissolvent composition of 5% CHX (w/v, pH 6, Pierre Fabre Santé) for group G, and $ZnCl_2$(Sigma-Aldrich) with a final concentration of 3.3 $mg.ml^{-1}$ pH 6.5, for group H.

ACTION OF MMP-3 ON PRETREATED DENTIN

Recombinant human MMP-3 (R&D Systems Inc., Minneapolis, MN, USA) was first incubated with aminophenylmercuric acetate

(1 mM, 1 h, 37°C) to achieve activation. Each demineralized and pretreated dentin block was incubated with the activated MMP-3 (1 µg/ml) in a 120 µl buffer containing 100 mM Tris pH 7.2; 0.15 M NaCl; $CaCl_2$ 5 mM; 0.05% w/v Brij 35, and 0.02% w/v NaN_3 (SIGMA, St Louis, MO, USA). Following 72 h incubation at 37°C, the supernatants were subjected to Western blot analysis to reveal the proteins released by MMP-3 treatment.

WESTERN BLOT ANALYSIS

Laemmli-buffer-diluted samples were electrophored on 4–20% w/v gradient gels (Mini-Protean® TGX™ gels, BioRad, Marne la Coquette, France) and separated proteins were transferred to a nitrocellulose membrane (Trans-Blot Transfer Medium, BioRad). The membrane was blocked with 5% v/v bovine serum and incubated overnight at 4°C with one of the following polyclonal antibodies: anti-DCN (LF 113), anti-BGN (LF 159) (kind gifts from Larry Fisher, NIH, Bethesda, MD, USA) used at 1 $mg.ml^{-1}$ and diluted at 1/1500; anti-DSP (LF 153) and anti-MEPE (kind gift from Peter Rowe, Kidney Institute, Kansas City, KS, USA) were used at 1 $mg.ml^{-1}$ and diluted at 1/700. After incubation with a peroxidase-conjugated swine anti-rabbit IgG at 1/10000 dilution for 1 h at room temperature, the membrane was developed by means of the Renaissance Western Blot Chemiluminescence Reagent Plus Kit (NEN Life Science, Boston, MA, USA). Chemiluminescent signals were digitalized and the relative intensity of the different bands was compared using ImageJ software (v1.45p, NIH).

STATISTICAL ANALYSIS

For each experiment, samples were performed in triplicate for each condition and repeated three times. The values found in each experiment were averaged, and the mean was used for comparison between groups. Intergroup comparison was performed by one-way analysis of variance with Bonferroni correction ($\alpha = 0.05$). If a statistical difference was detected, a *post hoc* non-pairwise multiple comparison (Scheffe's test) was used to identify differences among the groups. Data are expressed as mean ± SD.

SCANNING ELECTRON MICROSCOPY ANALYSIS (SEM)

To investigate whether the different tested pretreatments have influence on demineralized dentin treated by MMP-3, SEM analysis was performed on the replicas of the dentin blocks. Dentin block surfaces were treated with phosphoric acid 40% (w/v, Onyx ™ Centrix, Shelton, CT, USA) for 20 s. After rinsing and gentle drying, a thick layer of dental adhesive resin (OptiBond® SoloPlus Kerr Corporation, Orange, CA, USA) was placed on dentin. After photopolymerization (Free light 3M ESPE, Seefeld, Germany), a layer of microhybrid flowable composite (Tetric EvoFlow®, Ivoclar Vivadent, Saint Jorioz, France) was placed and photopolymerized. The dentin block was dissolved with HCl 8N during 36 h. Replica surfaces were rinsed with flow water, air-dried, glued with carbon adhesive discs on aluminum stubs, sputter-coated (SC5OO, BIO-RAD Laboratories, Hercules, CA, USA) with palladium/gold (20 mA, 0.1 Tor, 2 min). The replicas were observed by SEM (EVO MA 10 SEM, Carl Zeiss, Oberkochen, Germany) equipped with tungsten gun and Everhart-Tornley secondary electron detector.

RESULTS

CLEAVAGE OF DENTIN MATRIX COMPONENTS BY MMP-3

Western blot analysis of supernatants retrieved from the samples pre-treated with mouthrinse and GSE, followed by MMP-3 treatment, did not reveal any detectable DCN band (**Figure 1**). In contrast, DCN was visualized as a large band around 60 kDa in the AmF, placebo, NaF, and PBS pre-treated groups, and as an additional weaker band at 130 kDa mainly detectable in the AmF and placebo pre-treated group (**Figure 1A**). BGN was detected as a single large band around 140 kDa in the supernatants of the samples pre-treated with AmF, NaF, placebo, and PBS, but not with the mouthrinse and GSE (**Figure 1B**). For both SLRPs, no band was detected in the CHX and $ZnCl_2$ pre-treated groups (**Figures 1A,B**). DSP was also released by MMP-3 and was observed as two bands around 150 and 75 kDa in the AmF, placebo, NaF and PBS pre-treated groups. No signal was detected in the mouthrinse, GSE and $ZnCl_2$ supernatants, whereas a smear labeling was seen with CHX pre-treatment (**Figure 1C**).

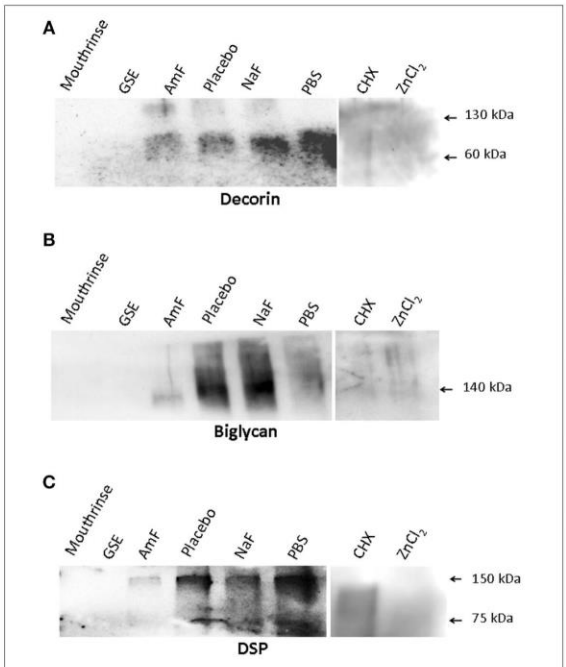

FIGURE 1 | Western blot analysis assessing the ability of the tested Mouthrinse to prevent NCP release. (A) DCN is detected as a main band at 60 kDa in the following groups: pre-treatment with amine fluoride (AmF), placebo, NaF, and PBS. An additional fainter band is seen at 130 kDa in the AmF and placebo groups. No band is observed in the Mouthrinse, GSE, CHX, and $ZnCl_2$ pre-treated groups. **(B)** BGN is detected as a large band around 140 kDa in placebo, NaF, and PBS pre-treated groups. This band appears fainter in the AmF group. No band is observed in the Mouthrinse, GSE, CHX, and $ZnCl_2$ pre-treated groups. **(C)** Dentin sialoprotein (DSP) is observed at 150 and 75 kDa in the AmF, placebo, NaF and PBS pre-treatment groups. A smeary signal is detected in the CHX pre-treated group whereas no band is present in the Mouthrinse, GSE, and $ZnCl_2$groups.

Quantification of the different bands after normalization with placebo is showed in **Figure 2**. In particular, release of DSP after pre-treatment with AmF was around 0.6 ($p < 0.05$), compared to release of DSP in placebo (set at 1), and release of BGN and DCN after pre-treatment with AmF were both around 0.4 ($p < 0.05$). Contrary to this, release of BGN and DCN after NaF pre-treatment were significantly higher compared to placebo (1.6 and 1.8, respectively, $p < 0.05$). At last, release of BSP and DCN after pre-treatment with PBS were significantly higher compared to placebo (1.8 and 2.8, respectively, $p < 0.05$) whereas release of BGN was significantly lower compared to placebo (0.9, $p < 0.05$). For MEPE, no signal was detected (data not shown).

EFFECT OF PRETREATMENTS ON DENTIN PENETRATION BY LOW VISCOSITY RESIN AFTER MMP-3 TREATMENT

Boukpessi et al. have previously shown that MMP-3 treatment, by removing some of the matrix components, improved the diffusion of low-viscosity resin into the dentin sub-surface (Boukpessi et al., 2008). Resin penetration into the dentin blocks was analyzed after MMP-3 treatment in the different groups (**Figure 3**). In the mouthrinse (**Figure 3A**) and GSE (**Figure 3B**) pretreatment groups, the tubules were not enlarged by the MMP-3 treatment and were not penetrated by the resin. Limited resin penetrations were observed in both fluoride pretreated groups (**Figures 3C,E**), but they were more pronounced in the NaF group. In contrast, a very deep penetration of the resin tags within the tubules was observed in the placebo and PBS groups (**Figures 3D,F**). With the two MMP inhibitors pretreatment, a faint disorganization of the dentin architecture was observed in CHX pretreated group (**Figure 3G**), whereas $ZnCl_2$ pretreated replicas showed a regular dentin surface (**Figure 3H**). Hence, the mouthrinse and its active components appear to limit the disorganization inside dentinal tubules induced by the MMP-3 action.

DISCUSSION

Our results show that dentin pretreatment with the tested mouthrinse, and to some extent with its active principles, limits dentin matrix disorganization induced by MMP-3, especially along the dentinal tubules (**Figure 4**). In this study, we used an *in vitro* model combining a demineralization process and a subsequent MMP-3 treatment in order to test MMP inhibitors on dentin matrix degradation (Boukpessi et al., 2008). MMP-3 has been identified in predentin (Hall et al., 1999) and in dentin (Boukpessi et al., 2008), where it localizes within the intertubular dentin, along the collagen fibrils (Mazzoni et al., 2009). *In vitro* human demineralized dentin has been extensively used as a model to study the caries process (Ten Cate et al., 1991). However, demineralized dentin represents a simplified model of the caries process (Ten Cate, 2001), as it does not take into account the degradation of the organic matrix by endogenous proteinases and/or by bacterial collagenases. Therefore, the selected model would be more relevant to mimic dentin matrix degradation and to test the effect of MMP inhibitors.

The reported roles for DCN and BGN include collagen stabilization, collagen fibrillogenesis, calcium binding potential, interaction with hydroxyapatite, as well as crystal growth inhibition. DCN is strongly expressed over the mineralized dentin (mainly inside the dentinal tubules) and in the predentin adjacent to the mineralization front, whereas BGN is mainly expressed in predentin during matrix formation and maturation (Mazzoni et al., 2012; Orsini et al., 2012). DSP, abundantly expressed in dentin and not detected in predentin, may be essential in the biomineralization process during the dentin formation (Mazzoni et al., 2012; Orsini et al., 2012). The prevention of these NCPs release may protect further matrix degradation by exposing the collagen fibers to more collagen-specific MMPs such as collagenases and gelatinases (Malla et al., 2008) which are also present in the dentin organic matrix and in the saliva (Tjaderhane et al., 1998).

Our Western blot results showed that the pretreatment of the demineralized dentin with either GSE or the mouthrinse inhibited the MMP-3-dependent release of two SLRPs and the SIBLING member DSP, a specific dentin NCP. It should be noted that the molecular weights of these glycoproteins released by MMP-3 are somewhat different from those of their purified forms. This may be due to their release either as dimers or as molecular forms still bound to fragments of other matrix components to which they are bound in the dentin. In fact, DCN, which

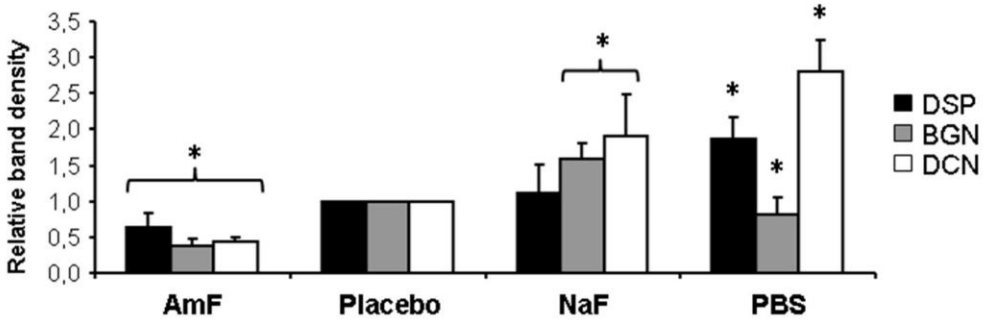

FIGURE 2 | Quantification of NCP release from Western blot analysis. Quantification of the different bands by ImageJ after normalization with placebo bands shows a significant weaker protein release in samples pre-treated with AmF (all proteins tested) and PBS (BGN), and a significant stronger release in NaF (BGN and DCN) and PBS (DSP and DCN) pre-treated groups. Black bars: DSP; gray bars: BGN; white bars: DCN. *$p < 0.05$ vs. placebo value.

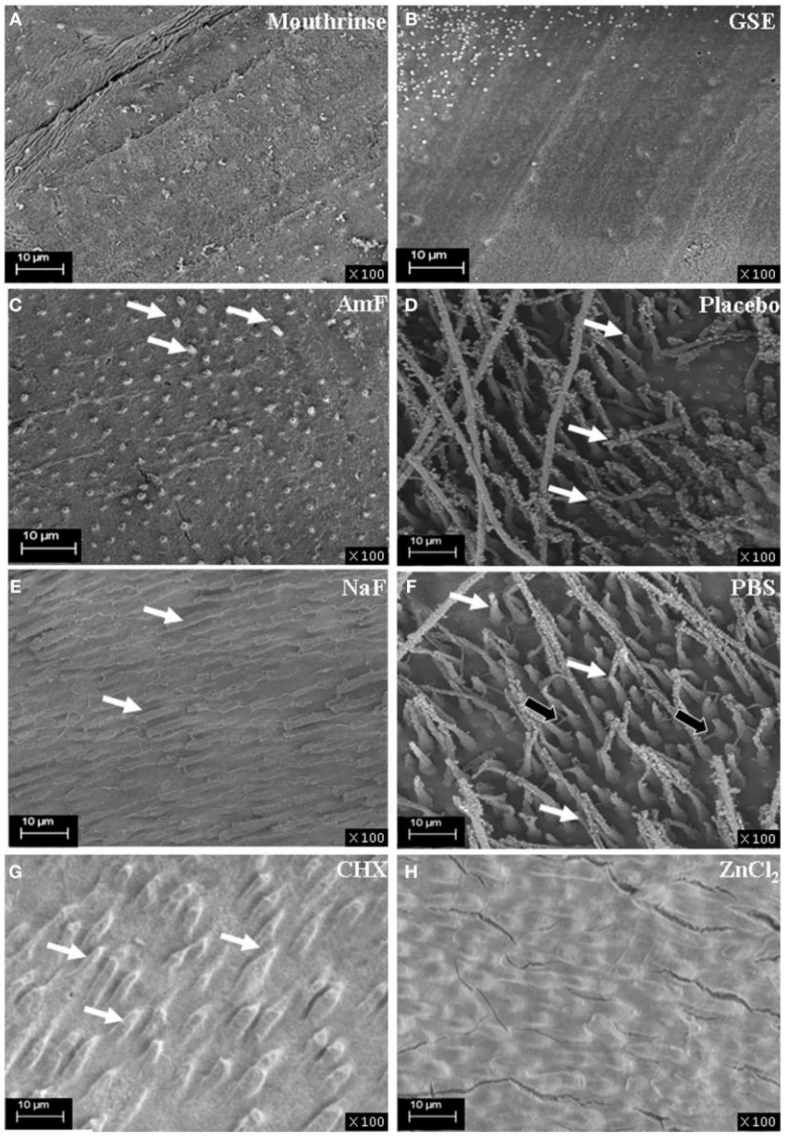

FIGURE 3 | SEM analysis of resin replicas of the demineralized, pre-treated and MMP-3 treated dentin blocks. The resin penetration is absent in the mouthrinse groups **(A)** and GSE **(B)** as no tubule is enlarged by the MMP-3 treatment and penetrated by resin; a regular dentin surface is observed. In the AmF group **(C)**, sparse penetrations of resin are seen (white arrows). In the NaF group **(E)**, mildly infiltrated dentinal tubules are visualized (white arrows). In contrast, a very deep penetration of the resin within the tubules (white arrows) is observed in the placebo **(D)**. In addition to a deep penetration of the tags within the tubules (white arrows) in the PBS group **(F)**, the filling of lateral secondary dentin tubules is observed (black arrows). A faint disorganization of the dentin architecture is seen in CHX pretreated samples (white arrows) **(G)**. $ZnCl_2$ pretreated replicas show a regular and smooth dentin surface **(H)**.

is reported in the literature at 45 kDa (Embery and Rolla, 1980), appeared on western blot at 60 kDa suggesting a protein complex. BGN, whose monomeric form is reported at 45 kDa (Embery et al., 2001), was detected as a band at 140 kDa. Interestingly, studies on the folding and stability of DCN and BGN implicated their organization as complex both in solution and as crystal structure, which is consistent with our result (Scott et al., 2006). For DSP, the molecular weights observed in the present study,

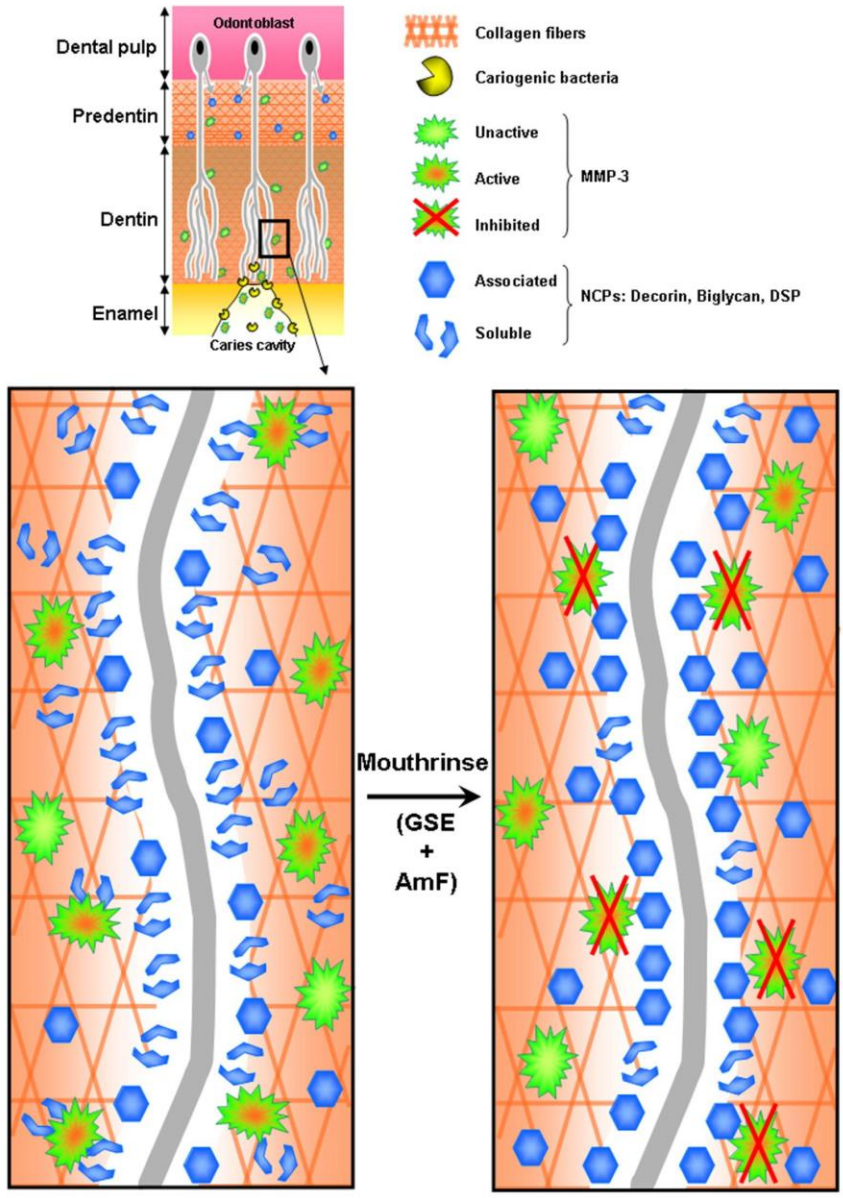

FIGURE 4 | Hypothetic representation of MMP-3 inhibition by the tested mouthrinse during the dentin carious process. Cariogenic bacteria present in the caries cavity release acids that demineralize the dentin matrix and activate host dentin or saliva-derived MMPs, including MMP-3. Activated MMP-3 then releases from the collagen scaffold several non-collagenous proteins (NCPs): DCN, BGN and some Sibling proteins. Mouthrinse, composed of GSE (Grape Seed Extracts) and AmF is an efficient MMP inhibitor that prevents the release of NCPs associated to the collagen fibers in the dentin.

150 and 75 kDa, are higher than those reported in the literature (95 and 53 kDa) (Ritchie et al., 1995). It can be proposed that MMP-3 releases these proteins by degrading their attachment to the dentin extracellular matrix.

The release of NCPs visualized by Western blot is in accordance with SEM exploration of the dentin surfaces showing that the pretreatment with the mouthrinse and GSE both inhibited the enlargement of the dentinal tubules by the enzymatic action of

MMP-3. This approach may be beneficial in terms of initial dentin caries prevention, but could also improve life expectancy of adhesive-resin interfaces. Indeed, previous studies have indicated that the use of MMP inhibitors prevent early degradation of resin-dentin bonds by endogenous MMPs (Sabatini and Patel, 2013) suggesting therefore that GSE could be used to treat the dentin surface of the cavity before the adhesive procedures. However, the consequences of this treatment on resin adhesion to the dentin surface still remain to be assessed by additional mechanical testing complemented by failure analysis.

GSE has been suggested to have an additional inhibitory effect on the demineralization process. It has been shown to inhibit the demineralization and/or to promote the remineralization of artificial root carious lesions *in vitro* under dynamic pH-cycling conditions (Xie et al., 2008). In addition, GSE treatment was also shown to increase the microhardness of the lesions in these conditions. Xie et al. suggested that GSE could contribute not only to the deposition of mineral on the superficial layer of the lesion, but may also interact with the organic portion of the root dentin through Proanthocyanidin-collagen interaction, stabilizing the exposed collagen matrix (Xie et al., 2008). Noteworthy, during the production of replicas, the 40% phosphoric acid dentin treatment opened the dentinal tubules only in placebo and PBS groups. This finding suggests that the mouthrinse and its active principles' pretreatment not only had an anti-MMP action but also modified the dentin surface accessibility (Bachelar-Sá et al., 2014). Along this line, dentin treatment with GSE has been reported to limit the matrix degradation by specific bacterial proteases through induction of collagen inter-microfibrilar cross-links by removing and replacing GAG by GSE (Bedran-Russo et al., 2008, 2011). At the concentration used in the present study (0.2%), it can be hypothesized that both an anti-MMP effect and a surface modification are achieved to protect the dentin matrix.

Regarding the effect of the other MMP inhibitors used, our results show that $ZnCl_2$ pretreatment was also effective in protecting dentin from cleavage by MMP-3 as there was no detectable release of matrix components, while with CHX pretreatment there was only a slight release of DSP. Interestingly, Toledano et al. have previously reported that these two MMP inhibitors prevented collagen degradation by endogenous MMPs in dentin beams pretreated with acidic solutions and incubated in artificial saliva (Toledano et al., 2012). However, only the $ZnCl_2$ pretreatment maintained a total inhibitory effect after a prolonged incubation period in the artificial saliva. Both approaches, conducted in different models, suggest that $ZnCl_2$ may be a more efficient MMP inhibitor over time than CHX. AmF, one of the active principles composing the tested mouthrinse, was a better inhibitor of MMP-3 in the present model than NaF as it decreased the release of DCN, BGN, and DSP from the matrix and prevented dentinal tubules enlargement. These protective effects on the dentin matrix were not observed with NaF pretreatment. Accordingly, Mei et al. compared MMP-inhibitory effects of several organic and inorganic fluorides by gelatin zymography and showed that the silver diamine fluoride, an organic fluoride, was the most efficient gelatinase inhibitor when compared with inorganic fluorides (Mei et al., 2012).

It can be hypothesized that the inhibition of NCP cleavage by MMP-3 inhibitors may prevent further matrix degradation by protecting the collagen fibers from collagen-specific MMPs such as collagenases and gelatinases. Indeed, PGs were initially reported as the major substrates of MMP-3. However, the situation may be more complex since PGs are bound to several other proteins in the extracellular matrix (Qin et al., 2006). For example, MMP-9 is linked to the core protein of Chondroitin sulfate proteoglycans, forming a pro-MMP-9-PG heteromer (Winberg et al., 2003; Malla et al., 2008). Although there is no literature reporting such complex in the dentin extracellular matrix, it cannot be excluded that the cleavage of this hetereomer by MMP-3 may release active MMP-9 (Malla et al., 2008) which could in turn express its gelatinolytic activity, exacerbating the dentin matrix degradation initiated by MMP-3.

In conclusion, the present study shows that the combination of GSE and AmF as in the evaluated mouthrinse limits dentin matrix degradation. This association may be promising in the prevention of caries. However, the procedure should be adapted to durations that are clinically relevant.

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4 Discussion

Tooth development results from reciprocal inductive interactions between the mesenchyme and the oral epithelium and proceeds through a series of well-defined stages (Ruch, Karcher-Djuricic, and Gerber 1973; Slavkin 1974; Catón and Tucker 2009; Miletich and Sharpe 2003; I Thesleff and Hurmerinta 1981; Mitsiadis and Luder 2011). Basement membrane degradation allowing a direct contact between pre-ameloblasts and pre-odontoblasts and their newly synthesized ECM appeared to be a crucial step of tooth development (Meckel, Griebstein, and Neal 1965; Cevc et al. 1980; Zeichner-David et al. 1995). EMMPRIN, a membrane glycoprotein also named CD147, has been shown to play an important role in the direct epithelial-mesenchymal interactions, as highlighted in the cancer field (Toole 2003). The expression of EMMPRIN in the developing tooth germs has been previously described (Schwab et al. 2007; Xie et al. 2010), increasing in the forming tooth germ from E14 to P1 (Xie et al. 2010). In this thesis, we confirmed that EMMPRIN was first expressed by pre-ameloblats and by the *stratum intermedium* at the early bell stage. At the late bell stage, EMMRIN labeling decreased on ameloblasts actively secreting enamel proteins, whereas it strongly increased on odontoblasts. Finally, this labeling disappeared in postsecretory ameloblasts whose function is to mature the enamel matrix.

However, at the beginning of this thesis, the *in vivo* role of EMMPRIN in tooth development and homeostasis was still unknown. By investigating mice KO for EMMPRIN, we showed that EMMPRIN, through the induction of several MMPs, may orchestrate the epithelial-mesenchymal cross-talk necessary for tooth formation, by enabling cleavage of the basement membrane and thus direct cell-cell interactions. Indeed in our mice we showed a delay in basement membrane degradation in KO mice when compared with WT. As a result, we observed a delay in ameloblast differentiation, especially detectable on transmission electron microscopy images (see **Figure 25**). As a consequence, MMP-3 and MMP-20 expression and activity were decreased and resulted in adults in decreased enamel volume and subtle abnormalities at the DEJ in both molars and incisors. It is noteworthy that we reported for the first time that EMMPRIN regulated the expression of MMP-20. As mice KO for MMP-20 display early enamel shedding and severe tooth alterations, our data suggest however that the quantity of MMP-20 expressed in the absence of EMMPRIN is sufficient to allow correct enamel maturation. Therefore, enamel volume was decreased in EMMPRIN KO mice but the maturation was rather-normal as indicated by nano-indentation experiments.

Surprisingly, in the tooth, EMMPRIN appeared to have no effect on MMP-2 and MMP-9 expression and activity. In several other processed such as corneal wound healing (Gabison et al, 2009), the absence of EMMPRIN was shown to alter gelatinase expression and activity. Our results therefore suggest that the action of EMMPRIN is organ-dependent.

Previous observations using Si-RNA experiments on mandibles in culture have indicated that EMMPRIN was involved in tooth morphogenesis (Xie et al, 2010). In our study, we showed that tooth phenotype was rather normal in EMMPRIN KO mice, which is not consistent with these previous *ex vivo* observations. We therefore propose that the direct effect of EMMPRIN on the epithelial-stromal interaction may be limited since it is only allowed between basement membrane degradation allowing direct cell contact and before calcified matrix deposition which constitutes cell barriers, hence limiting EMMPRIN's action (**Figure 34**).

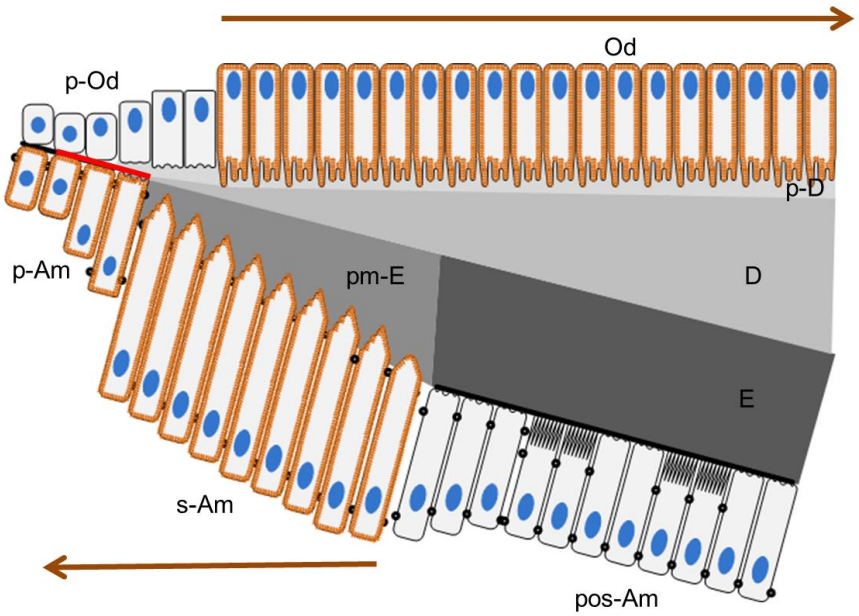

Figure 34: **Recapitulative schema proposing the role of EMMPRIN in tooth formation. At early bell stage, EMMPRIN is expressed by pre-ameloblast (p-Am) and may orchestrate basement membrane degradation (black line) to allow direct contact with pre-odontoblast (p-Od), which is mandatory for the final cell differentiation. At secretory stage, both secreting ameloblasts (s-Am) and odontoblasts (Od) highly express EMMPRIN. This expression may enhance MMP-20 synthesis by ameloblasts allowing for early enamel maturation. At the enamel maturation stage, post-secretory ameloblasts (pos-Am) lose their EMMPRIN expression. The arrows indicate EMMPRIN expression by cells. The red line schematizes the time window where a direct effect of EMMPRIN is allowed by a direct cell contact. D: dentin; pD: predentin; pm-E: premature enamel; E: enamel.**

EMMPRIN has been shown to be involved in the repair process of different injured tissues through MMP induction and increase in myofibroblast contractile activity (Gabison, Mourah, et al. 2005; Huet, Vallée, et al. 2008). We investigate for a potential role of EMMPRIN in the pulp dentin repair process by comparing the healing of injured pulps of EMMPRIN KO and WT mice. The repair process seems to be improved in KO mice but these preliminary data must be repeated and supported by histological analysis.

MMPs have been suggested to contribute to dentin caries progression and the hypothesis that MMP inhibition would affect dentin caries progression is clinically interesting. This hypothesis was sustained by *in vivo* studies in rat caries models where dentin caries progression was delayed by intra-oral administration of chemical MMP inhibitors, modified tetracylines and zoledronate (Sulkala et al. 2001; Tjäderhane et al. 1999). The MMP-inhibitory effects of Grape-seed extracts (GSE) suggest that these natural substances could be effective in inhibiting dentin caries progression. We therefore evaluated the capacity of these natural agents incorporated in a mouthrinse to prevent the degradation of demineralized dentin matrix by MMP-3. In this study, we selected MMP-3 because we have previously shown that this enzyme was the only MMP among those tested (MMP-2, MMP-3 and MMP-9) that was able to degrade several NCPs (Boukpessi et al., 2008), known to be associated to the collagen fibers in the dentin (Orsini et al., 2006). The removal of these NCPs can then permit further matrix degradation by exposing the collagen fibers to more collagen-specific MMPs such as collagenases and gelatinases (Malla et al., 2008) which are also present in the dentin organic matrix and in the saliva (Tjaderhane et al., 1998).

Our results show that dentin pretreatment with the tested mouthrinse, and with its active principles, inhibited the release by MMP-3 of several NCPs, namely decorin, biglycan and DSP from the matrix and the disorganization along the dentinal tubules induced by MMP-3. We therefore hypothesized that the inhibition of NCP cleavage by GSE may prevent further matrix degradation by protecting the collagen fibers from collagen-specific MMPs such as collagenases and gelatinases. Indeed, PGs were initially reported as the major substrates of MMP-3. However, the situation may be more complex since PGs are bound to several other proteins in the ECM (Qin *et al*., 2006), and once the degradation of the dentin ECM is initiated, it may be more susceptible to further degradation by other proteases. Interestingly, amine fluorides appear to have MMP inhibitory properties at a lesser degree than GSEs but at

a higher level than NaF. This information is clinically relevant, fluorides being recognized as the most efficient tools for preventing dental caries. However, it requires further investigations to be confirmed.

As general conclusion, proteases and their regulator such as EMMPRIN appear to have a major role in the formation, pathologies and repair of the tooth. Therefore their understanding opens several therapeutic issues, especially for the prevention and the treatment of dentin caries.

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6 Annexe 1

Benjamin Salmon, Claire Bardet, **Mayssam Khaddam**, Jiar Naji, Benjamin R. Coyac, Brigitte Baroukh, Franck Letourneur, Julie Lesieur, Franck Decup, Dominique Le Denmat, Antonino Nicoletti, Anne Poliard, Peter S. Rowe, Eric Huet, Sibylle Opsahl Vital, Agne`s Linglart, Marc D. McKee, Catherine Chaussain. 2013. "MEPE-Derived ASARM Peptide Inhibits Odontogenic Differentiation of Dental Pulp Stem Cells and Impairs Mineralization in Tooth Models of X-Linked Hypophosphatemia"

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MEPE-Derived ASARM Peptide Inhibits Odontogenic Differentiation of Dental Pulp Stem Cells and Impairs Mineralization in Tooth Models of X-Linked Hypophosphatemia

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Abstract

Mutations in *PHEX* (phosphate-regulating gene with homologies to endopeptidases on the X-chromosome) cause X-linked familial hypophosphatemic rickets (XLH), a disorder having severe bone and tooth dentin mineralization defects. The absence of functional PHEX leads to abnormal accumulation of ASARM (acidic serine- and aspartate-rich motif) peptide – a substrate for PHEX and a strong inhibitor of mineralization – derived from MEPE (matrix extracellular phosphoglycoprotein) and other matrix proteins. MEPE-derived ASARM peptide accumulates in tooth dentin of XLH patients where it may impair dentinogenesis. Here, we investigated the effects of ASARM peptides *in vitro* and *in vivo* on odontoblast differentiation and matrix mineralization. Dental pulp stem cells from human exfoliated deciduous teeth (SHEDs) were seeded into a 3D collagen scaffold, and induced towards odontogenic differentiation. Cultures were treated with synthetic ASARM peptides (phosphorylated and nonphosphorylated) derived from the human MEPE sequence. Phosphorylated ASARM peptide inhibited SHED differentiation *in vitro*, with no mineralized nodule formation, decreased odontoblast marker expression, and upregulated MEPE expression. Phosphorylated ASARM peptide implanted in a rat molar pulp injury model impaired reparative dentin formation and mineralization, with increased MEPE immunohistochemical staining. In conclusion, using complementary models to study tooth dentin defects observed in XLH, we demonstrate that the MEPE-derived ASARM peptide inhibits both odontogenic differentiation and matrix mineralization, while increasing MEPE expression. These results contribute to a partial mechanistic explanation of XLH pathogenesis: direct inhibition of mineralization by ASARM peptide leads to the mineralization defects in XLH teeth. This process appears to be positively reinforced by the increased MEPE expression induced by ASARM. The MEPE-ASARM system can therefore be considered as a potential therapeutic target.

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Introduction

Mineralization is regulated by a number of determinants, including small molecules such as pyrophosphate, and proteins (including enzymes and hormones), that act either locally in the extracellular matrix and/or systemically to regulate calcium and phosphate metabolism. Currently, the complex links between these molecules are not well understood. Of note for tooth dentin

(unlike bone) is that dentin is not involved in the regulation of the calcium and phosphate metabolism, and dentin is not remodeled. Thus, it is a tissue showing a permanent chronological record of disease that may be useful in deciphering these complex molecular interactions and mechanisms of mineralization.

Skeletal mineralization is severely impaired in patients with mutations in the genes encoding mineralization-regulating proteins [1]. Genetic disorders, such as X-linked familial hypophosphatemic rickets (XLH), may affect both bone and tooth mineralization [2]. Mineralization defects in XLH appear in children as rickets with severe skeletal deformities and as osteomalacia and bone or join pain in adults. Patients with XLH frequently present spontaneous dental abscesses, without any history of tooth trauma or decay [3,4,5]. Despite the fact that teeth of patients with XLH seem visibly normal, radiography commonly reveals thin enamel and radiolucent dentin – the latter being associated with unusually large pulp chambers. In XLH, dentin mineralization is impaired [6,7,8], with the defect appearing as unmerged, persistent mineralization foci (calcospherites) surrounded by extensive interglobular regions of unmineralized dentin matrix [6]. These abnormalities may lead to rapid pulp necrosis associated with periapical bone infection. Within the pathologic, unmineralized interglobular dentin matrix, many of the extracellular matrix (ECM) components, including noncollagenous proteins (NCPs), are degraded [7,9,10,11]. Among them, degraded members of the SIBLING family (small integrin-binding ligand N-linked glycoproteins) have been identified in XLH dentin [12,13]. This family of proteins shares a common acidic serine- and aspartate-rich motif (ASARM), which has a key role in the mineralization process [14,15,16,17,18,19].

Among the SIBLING proteins, MEPE (matrix extracellular phosphoglycoprotein) has been shown to be involved in pathologic processes associated with hypophosphatemic rickets [16,20,21,22,23]. MEPE is expressed by both osteoblasts and odontoblasts and is normally protected from cleavage by a protein-protein interaction between its ASARM motif and PHEX (phosphate-regulating gene with homologies to endopeptidases on the X-chromosome) [24,25] – a transmembrane endopeptidase. More than 80% of patients with familial hypophosphatemic rickets carry a mutation in the *PHEX* gene [26,27,28,29]. In these patients, unprotected MEPE is exposed to pathologic cleavage by local proteinases such as cathepsin B, releasing ASARM peptides into the ECM and the circulation [24,30,31]. Moreover, since ASARM is normally a substrate for the enzymatic activity of PHEX [32,33], the lack of functional PHEX in XLH patients results in the accumulation of these proteinase-resistant peptides that are thought to lead to mineralization defects in bone and tooth ECM [12,14,23,34]. Other mineralization-regulating SIBLING proteins, such as OPN and DMP1 (dentin matrix protein 1), as well as the ASARM-containing peptides derived from their cleavage, may also be involved in the mineralization pathology [14,32,35,36]. Previous *in vitro* studies have shown that mouse-derived bone marrow stromal cells (BMSCs) and osteoblasts treated with phosphorylated MEPE-derived (and OPN-derived) ASARM synthetic peptides failed to properly mineralize their ECM [14,32,33,37,38].Since human teeth are severely affected by the disease [3,6], this study aimed to investigate *in vitro* and *in vivo* the effects of the MEPE-derived ASARM peptide on tooth dentin mineralization. We used pulp progenitor stem cells from human exfoliated deciduous teeth (SHEDs), as we and others have shown that deciduous teeth are mostly affected in patients with XLH [3,7,8,30]. These cells were induced toward an odontogenic differentiation program using a cell culture collagen/tooth slice 3D scaffold model. In parallel, we implanted MEPE-derived ASARM peptides into surgically injured pulp of rat molars [39], and their effects on reparative dentin formation were evaluated. From these *in vitro* and *in vivo* studies reported here, we demonstrate that phosphorylated MEPE-derived ASARM peptide inhibits dentin mineralization, disturbs odontoblast differentiation and dramatically upregulates MEPE expression. This ASARM peptide – previously identified and shown to accumulate in dentin from patients with XLH [12] – therefore appears to be a key molecule in the pathogenesis of tooth dentin abnormalities as observed in XLH patients.

Materials and Methods

Human Teeth

Teeth were obtained from the Dental Department of Hopitaux Universitaires Paris Nord Val de Seine, AP-HP, France. Deciduous teeth were collected after trauma or after exfoliation from three healthy young children (3–7 years of age). Permanent third molars were obtained after extraction according to an orthodontic treatment plan. All teeth were collected with informed and oral consent from the patients and the parents according to ethical guidelines set by the French law (Loi Bioéthique n°2004–800) and with a special authorization for our team (n°DC-2009–927, Cellule Bioéthique DGRI/A5, direction générale pour la recherche et l'innovation, Ministère de l'enseignement supérieur et de la recherche, Paris, France).

Synthetic ASARM Peptides

Phosphorylated ASARM (p-ASARM with 3 phosphoserine residues) and nonphosphorylated ASARM (np-ASARM) peptides were synthesized according to the human MEPE-derived sequence as previously reported [14], and were RDDSSESSDSG-S(PO_3H_2)SS(PO_3H_2)ES(PO_3H_2)DGD and RDDSSESSDSGSS-SESDGD, respectively.

Cell Culture

Culture of pulp stem cells from human exfoliated deciduous teeth (SHEDs) were established as previously reported [40]. Briefly, after decontamination with povidone-iodine solution (Betadine, Meda Pharma, France), teeth were sectioned longitudinally and exposed pulp tissues were collected and enzymatically digested with type I collagenase (3 mg/ml; Worthington Biochem, Freehold, NJ, USA) and dispase (4 mg/ml; Boehringer Mannheim, Germany). Single-cell suspensions were obtained by passing the digested tissues through a 70 µm cell strainer. Cells were then seeded at a density of $10^4/cm^2$, and the cultures were maintained with Dulbecco's Modified Eagle Medium 1 g/L D-Glucose (DMEM; Invitrogen, Grand island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1% penicillin/streptomycin (PS; Invitrogen), at 37°C with 5% CO_2. The medium was refreshed the next day after initial cell attachment and then after at 3 times per week. Cells were detached by trypsinization at 70–80% confluence (0.25% trypsin EDTA solution Sigma-Aldrich, St. Louis, MO, USA) and either re-plated at the same density or frozen at −80°C. For all experiments, SHEDs were used between passages 2 and 4. Their SHED phenotype (CD90+, CD29+, CD44+, CD45−, CD73+, CD105+, CD146+, and 10% of STRO-1+) was confirmed by polychromatic flow cytometry analysis (LSRII, Becton Dickinson, NJ; antibodies from Biolegend, CA, BDBiosciences, NJ and eBioscience, CA) (Fig. S1).

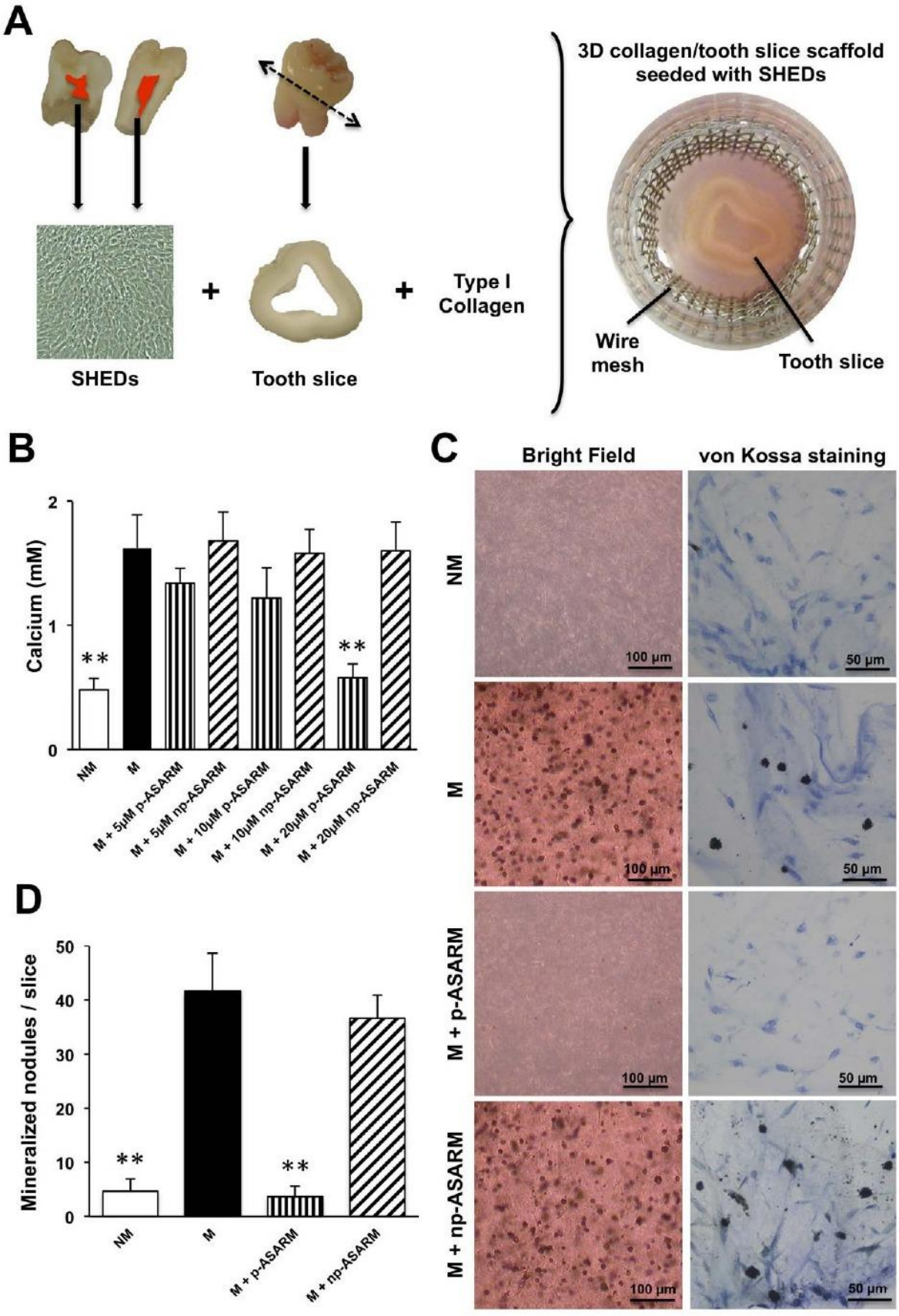
A
SHEDs
Tooth slice
Type I Collagen
3D collagen/tooth slice scaffold seeded with SHEDs
Wire mesh
Tooth slice
B
Calcium (mM)
NM
M
M + 5μM p-ASARM
M + 5μM np-ASARM
M + 10μM p-ASARM
M + 10μM np-ASARM
M + 20μM p-ASARM
M + 20μM np-ASARM
C
Bright Field
von Kossa staining
NM
M
M + p-ASARM
M + np-ASARM
100 μm
50 μm
D
Mineralized nodules / slice
NM
M
M + p-ASARM
M + np-ASARM

Figure 1. Inhibition of mineralization by phosphorylated MEPE-ASARM peptide in a 3D collagen/tooth slice culture model. A: Schematic of the 3D culture model. Stem cells from human deciduous exfoliated teeth (SHEDs) were obtained for cell cultures studies from the pulp of caries-free human third molars. Passaged cells (10^6 SHEDs) seeded into a type I collagen gel scaffold were applied to a human tooth slice with a pulp chamber cavity to mimick the tooth/dentin environment, all of which were supported peripherally by a steel wire mesh to minimize collagen gel contraction. **B:** Calcium content in the cell/matrix layer determined by flame atomic absorption spectrometry of cultures maintained in NM or M conditions in the absence or presence of p-ASARM or np-ASARM at a concentration of 5, 10 or 20 μM for 21 days. Calcium content significantly decreased in the presence of 20 μM of p-ASARM while the nonphosphorylated form of the peptide had no effect on calcium content of the cultures. **C-D:** SHED cultures were maintained in nonmineralizing (NM) or mineralizing (M) conditions (see Materials and Methods) in the absence or presence of 20 μM phosphorylated (p-ASARM) or nonphosphorylated (np-ASARM) peptide for 21 days. Mineralized nodules in the extracellular matrix were clearly visible by light microscopy after von Kossa staining in the M and M+np-ASARM. Quantification of mineralized nodules (**D**) shows that they were essentially undetectable in presence of the p-ASARM peptide, whereas about 40 nodules were counted per slice in the M and M+np-ASARM samples. $n = 3$, error bars +/− SD, ** indicates significant difference ($p<0.01$) relative to mineralizing condition without peptide.
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Tooth Slice Preparation

Caries-free human third molars extracted for orthodontic treatment purposes were collected from healthy young adults (18–25 years of age) with informed and oral consent of the patient, according to ethical guidelines set by the French law (see above). One-mm-thick tooth slices were prepared as previously described [41]. After disinfection with 70% ethanol, teeth were transversely sectioned at the cervical region using a diamond saw blade under cooling with sterile phosphate-buffered saline (PBS, Invitrogen) to obtain 1-mm-thick dentin slices. The pulp tissue was completely removed and tooth slices were immersed in 0.05% sodium hypochlorite for 10 s, then washed with sterile 1× PBS.

3D Culture Model Seeded with SHEDs

Tooth slices (one slice per well) were placed into wells of a 24-well culture plate for the cell culture studies. To provide a 3-dimensional (3D) cell culture environment, the empty pulp chamber was filled with 1 ml of polymerizing collagen gel prepared by mixing 10% (v/v) of ice-cold 10× DMEM (Sigma-Aldrich), 10% (v/v) of $NaHCO_3$ (37 g/l), 50% (v/v) of type I collagen solution (2 mg/ml) and 30% (v/v) of SHEDs in suspension at 10^6 cells/ml. To avoid adhesion and migration of the cells from the scaffold to the bottom of a conventional cell culture plate, cell culture-suspension plates were used (Cellstar, Greiner bio-one, Basel, Switzerland). The type I collagen used was extracted from rat tail tendons as previously described [42]. A sterile, stainless steel grid (Weber Metaux, Paris, France) was placed along the edge of the well to prevent gel contraction.

Induction of the Odontoblast Differentiation Program

To induce the odontoblast differentiation program in the SHEDs, cells seeded into the 3D collagen/tooth slice scaffold (Fig. 1A) were cultured up to 21 days at 37°C with 5% CO_2, 10% FBS, and 1% penicillin/streptomycin in supplemented DMEM in the presence of 10 mM ß-glycerophosphate (a source of phosphate for mineralization), 50 μg/ml ascorbic acid (promotes collagen secretion and matrix assembly), and of 10^8 M dexamethasone (which induces expression of biomineralization markers) − this is termed the mineralization condition (M) in our study [43]. Using this mineralization condition, either p-ASARM or np-ASARM (at concentrations of 5, 10, 20 μM for both) were added to the cultures during media changes 3 times per week. The negative control, termed the nonmineralizing condition (NM), consisted of DMEM cultures supplemented with 10% FBS and 1% PS. In all cases, medium was changed 3 times a week. Samples were collected at days 7, 14 and 21. At each time point and for each condition, cell morphology was observed using an inverted microscope (Olympus CKX41, Tokyo, Japan). All experiments were repeated in triplicate with cells from the same donor.

Mineralization Assays

For assessing mineralized nodule formation after 3 weeks of culture, SHED-seeded 3D collagen/tooth slice scaffolds were fixed overnight at 4°C in 4% paraformaldehyde (PFA) solution and embedded in methyl methacrylate (Merck, Darmstadt, Germany) without any demineralization process [44]. Nine-micrometer-thick tissue sections were prepared, and mineralization in the ECM was revealed by von Kossa staining carried out by applying a 5% silver nitrate solution (Sigma) to the sections. Sections were then counterstained with toluidine blue. Mineralized nodules were counted in 30 randomly analyzed fields for each condition at 20× magnification and results are expressed as the number of nodules/tooth slice scaffold.

Table 1. Primer design for RT-PCR analysis.

	5′ –3′ Forward primer	5′ –3′ Reverse primer	Product
MEPE	GAGTTTTCTGTGTGGGACTACT	GCTTTGCTTAGTTTTCTCAGTC	86-bp
NM_020203.2			
DSPP	GAGGAGATGCTTCTTATAACTCTG	GTGCTATCACTGTCATCATCTT	91-bp
NM_014208.3			
Osteocalcin	GCACCCTTCTTTCCTCTT	GGAGTTTATTTGGGAGCAG	95-bp
NM_199173.3			
COL1	TGACTGGAAGAGTGGAGAGTA	TCTTGCTGATGTACCAGTTCT	149-bp
NM_000088.3			
ALPL	AACTGATGTGGAGTATGAGAGTG	GAAGTGGGAGTGCTTGTATCT	112-bp
NM_000478.4			

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Table 2. Clinical and biological characteristics of the patients with XLH at the time of tooth extraction.

Patients with XLH	**1**	**2**
Sex	M	F
Age (yr)	3	15
(at the time of tooth collection)		
Treatment	–	+
Age at onset (yr)	*not yet started at the time*	3.3
Duration (yr)	*of tooth collection*	10
Unalfa® (μg/day)	–	1.25
Phosphates (mg/day)	–	2100
Compliancy	–	moderate
Signs of rickets		
Leg bowing	+++	+
Height (SD)	−3	−2.1
Serum P (mmol/l)	0.63	0.58
Alkaline phosphatase (IU/L)*	498	523
Dental status	vital	vital
DMFT or dft**	7	2
Collected tooth	61,71	38, 48
Type	deciduous	permanent
Pulp status	vital	vital
Reason of extraction	(trauma)	(orthodontic treatment
		plan)

*(normal range is inferior to 300 IU/L).
**DMFT: Decayed Missing Filled permanent Teeth; dft: decayed filled deciduous teeth.
doi:10.1371/journal.pone.0056749.t002

Quantification of calcium content extracted from mineral deposits within the cell/matrix layer after 21 days of culture was also assessed. For this, the 3D collagen scaffolds seeded with the SHEDs were gently removed from the tooth slice, rinsed twice with PBS, and decalcified with 0.5 M HCl for 1 hr at room temperature under constant agitation. After centrifugation at 2.150 g for 10 min, the calcium content in the supernatant was determined by the flame atomic absorption spectrometry method (AAnalyst, Perkin Elmer, Waltham, MA, USA).

Immunohistochemistry

After 3 weeks of culture, SHED-seeded 3D collagen/slice scaffolds were fixed overnight at 4°C in 4% paraformaldehyde (PFA) solution, and embedded in methyl methacrylate (Merck, Darmstadt, Germany) without any demineralization process [44]. Nine-micrometre-thick tissue sections were prepared and endogenous peroxidases were blocked by incubation of the sections with 5 mM periodic acid at room temperature for 10 min. After extended rinsing in PBS, background activity was blocked at room temperature for 90 min using 1% bovine serum albumin (BSA) in PBS. Mouse monoclonal antibodies for osteocalcin (ab13420, Abcam, Cambridge, UK) and DSP (dentin sialoprotein, LFMb-21, Santa Cruz Biotechnology, CA, USA), and rabbit polyclonal antibodies for the MEPE mid-region and PHEX [14] were used all at a 1/200 dilution. Sections were treated in a moist chamber overnight at 4°C and further incubated for 90 min with a polyclonal goat anti-mouse immunoglobulin/HRP conjugate (Dako, Glostrup, Denmark) or with a polyclonal swine anti-rabbit immunoglobulin/HRP conjugate (Dako). Peroxidase visualization was obtained over 45 s in a dark chamber using 3.3′-diaminobenzidine tetrahydrochloride (Fast Dab, Sigma-Aldrich). The sections were counterstained with hematoxylin staining. Controls were carried out by omitting the primary antibody to exclude nonspecific binding. For PHEX, a goat anti-rabbit immunoglobulin-G Alexa Fluor 488 was used at a dilution of 1/200 (Sigma-Aldrich) in a dark-chamber, and nuclei were counterstained with dapi (Invitrogen). Microscopic images were merged using Image J software ver1.45p (public domain image processing and analysis software developed at the National Institutes of Health, Bethesda, MD, USA).

Scanning Electron Microscopy

After 3 weeks of culture, 3D collagen/tooth slice scaffolds were fixed overnight in 1% PBS-buffered glutaraldehyde at 4°C and processed for scanning electron microscopy (SEM). Samples were fractured in liquid nitrogen and carbon- or gold-sputtered surfaces were observed in a scanning electron microscope (Cambridge S260 SEM, UK) equipped with an Everhart-Tornley and back-scattered electron detector (BSE) and with an energy-dispersive X-ray spectrometer (EDX) for X-ray microanalysis (Bruker Axs, WI, USA) with Esprit2 software.

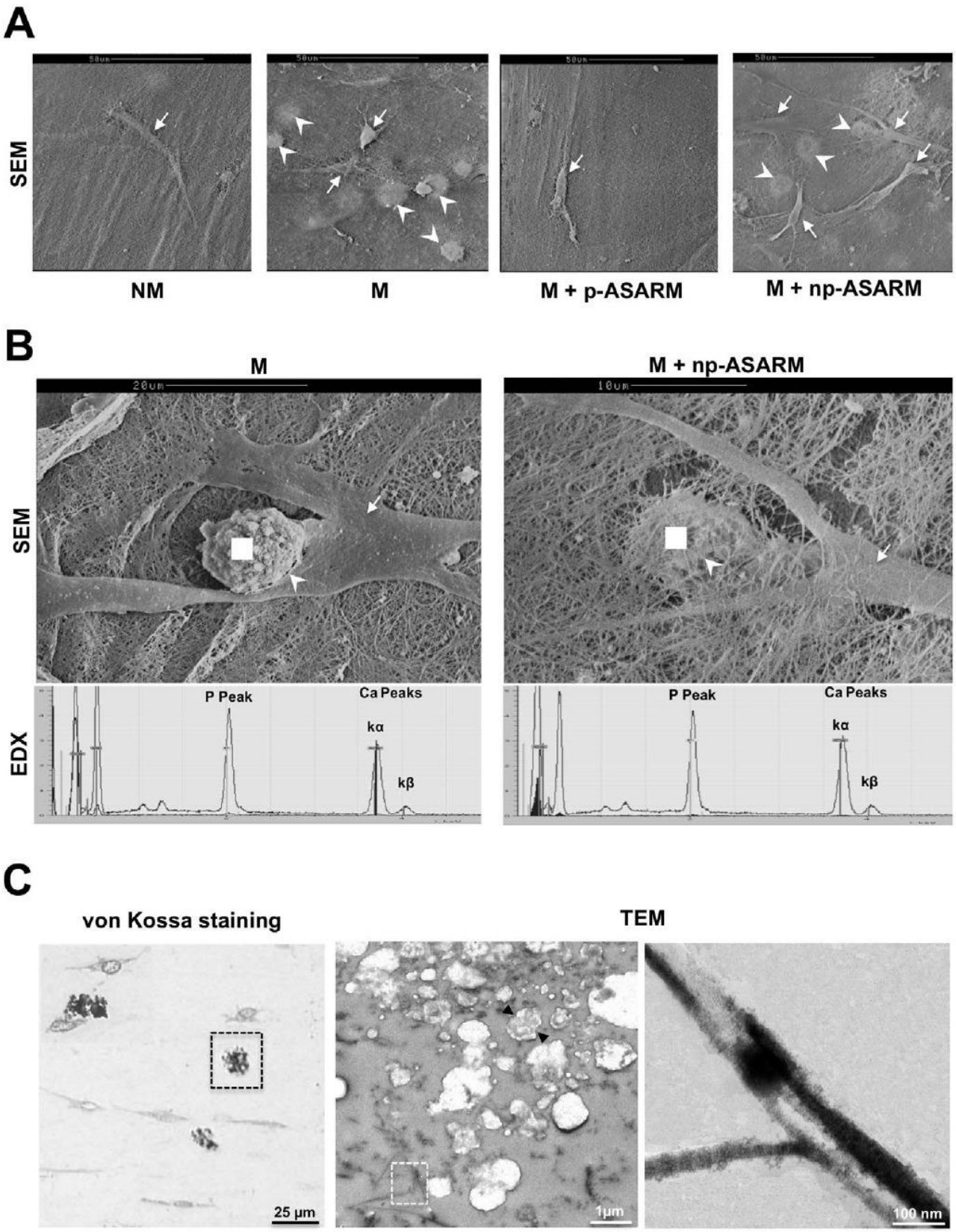

Figure 2. Light and electron microscopy of the cells, matrix and mineral in 3D SHED cell cultures. SHED cell cultures maintained in nonmineralizing (NM) or mineralizing (M) conditions in the absence or presence of 20 µM of either phosphorylated (p-ASARM) or nonphosphorylated

(np-ASARM) ASARM peptide for 21 days were visualized by light microscopy and by scanning (SEM) and transmission (TEM) electron microscopy. **A,B:** SEM reveals SHEDs (arrows) well integrated into the collagen scaffold. Mineralization of the cultures appears as nodules within the collagen scaffold (arrowheads) only in the M and the M+np-ASARM conditions. Energy-dispersive X-ray spectroscopy (EDX) for compositional microanalysis of the nodules (performed at the white square) shows major spectral peaks for calcium (Ca) and phosphorus (P) with an acquired Ca/P ratio of 1.67+/−0.05 in both mineralizing conditions where nodules appeared. **C:** Light microscopy (left panel) and TEM (center and right panel) of the mineralized cultures (M and M+np-ASARM). Mineralized nodules (black box, left panel) are often in close proximity to the SHED cells, and consist of aggregates of multiple, smaller mineralization nodules (arrowheads) and occasional mineralized collagen fibrils (white box center panel, and right panel).
doi:10.1371/journal.pone.0056749.g002

Light Microscopy and Transmission Electron Microscopy

Light microscopy and transmission electron microscopy (TEM) were used to analyze cells and mineralization of the 3D collagen/tooth slice scaffolds. Scaffolds were fixed overnight at 4°C in 4% paraformaldehyde and embedded in methyl methacrylate (Merck, Darmstadt, Germany). One-µm-thick survey sections were cut for light microscopy using a Reichert Ultracut S microtome, and sections were stained with von Kossa reagent (5% silver nitrate solution, Sigma) to reveal mineralization sites within the scaffold, and then counterstained with toluidine blue. Thin sections (90 nm) were cut and placed onto an electron microscopy grid followed by conventional staining with uranyl acetate and lead citrate at room temperature, and some sections were additionally subsequently stained with von Kossa reagent (5% silver nitrate solution, Sigma). Mineralized regions of the scaffolds were examined, and images recorded, using a Leitz DMRBE optical microscope equipped with a Sony DXC-950 CCD camera, and using a JEOL 1011 TEM operating at 80 kV and equipped with a GATAN Erlangshen camera 1000.

RT-PCR Analysis for Gene Expression

The mineralizing potential of the SHED cultures treated with the ASARM peptides was investigated by analyzing the expression of type I collagen α-chain (*COLI*), dentin sialophosphoprotein (*DSPP*), *MEPE*, *PHEX*, osteocalcin, and tissue-nonspecific alkaline phosphatase (*ALPL*) genes after extraction of total RNA from the cultures after 7, 14 and 21 days using RNeasy Mini kit (Qiagen, Hilden, Germany). 300 ng of total RNA was reverse-transcribed with Superscript II (Gibco, Invitrogen) using oligodT. cDNA was amplified with specific primers (Eurofins mwg operon, Ebersberg, Germany) in a SYBR® Green PCR Master Mix (Roche, Basel, Switzerland). Each primer pair was chosen for best efficiency and controlled for specificity (Table 1). Quantitative real-time PCR (qPCR) analysis was carried out using LigthCycler 480 [45]. The PCR cycles consisted of an initial pre-incubation step (95°C for 5 min), followed by 45 cycles of amplification (95°C for 5 s, 55°C for 5 s, 70°C for 10 s). Relative gene expression levels were estimated using the deltaCq method [46]. *GAPDH* and *ubiquitine C* (*UBC*) were determined using GeNorm software on a set of 5 standard housekeeping genes and were used as housekeeping genes for the rest of the study. Results were expressed as mean ± SD of relative mRNA level for each time point and for the different conditions.

Total Protein Extraction and Western Blot Analysis

At 7, 14 and 21 days, the collagen matrices seeded with SHEDs were gently removed from the tooth slices and processed for total protein extraction using ice-cold extraction buffer (each collagen scaffold was placed in 100 µl buffer: 50 mM Tris-HCl, pH 7.5, containing 5 mM EDTA, 150 mM NaCl and 0.2% Triton X-100), supplemented with 1/100 Protease Inhibitor Cocktail Set V EDTA free (Calbiochem, La Jolla, CA, USA). The resulting homogenates were briefly sonicated on ice, cleared by centrifugation at 10,000 g for 10 min at 4°C and stored at −80°C. Homogenates (10 µg) were subjected to 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad). Membranes were incubated overnight at 4°C with monoclonal anti-DSP (1/2000; LFMB-21, Santa Cruz) and anti-osteocalcin (1/1000; ab13420, Abcam) antibodies, or polyclonal anti-MEPE mid-region and anti-PHEX antibodies at 1/2000 dilution [14]. Membranes were incubated with a peroxidase-linked swine anti-rabbit secondary antibody (1/10,000) for 2 hrs at room temperature, then rinsed and followed by enhanced chemiluminescence detection of bound primary antibodies (Roche Diagnostics, Meylan, France). As a control for protein loading, membranes were carefully washed in stripping buffer (Pierce Chemical, Rockford, IL, USA) and processed with a monoclonal mouse anti-α tubulin antibody (clone B-5-1-2, Sigma). Quantification was performed using Image J software.

Implantation of ASARM Peptides in a Rat Pulp/Dentin Injury Model

Affi-Gel agarose beds (Bio-Rad Laboratories) were soaked in a solution of p-ASARM or np-ASARM dissolved in PBS (2 µg/µl) for 2 hrs, and dried overnight at 37°C. Beads soaked with PBS were used as a control. The beads were implanted into the pulp of the upper first molar of 9 rats (6 week-old, OFA/SD Charles River, Lyon, France) using a surgical procedure previously described [47]; this study was designed according to the ARRIVE guidelines and approved by the Animal Care Committee of the University Paris Descartes (agreement CEEA34.CC.016.11, Comité d'éthique pour l'expérimentation animale n° 34, Paris, France). Each animal received two of the three compared treatments on the left and right first maxillary molars. Treatments were then analyzed between animals using the molar as the statistical unit. Briefly, after gingival electrosurgery, 18 half-moon-shaped class V 0.5 mm cavities were prepared on the mesial aspect of the first upper molar using a 0.2-mm-diameter round bur (E0123, Dentsply Maillefer, Ballaigues, Switzerland). Pulp exposure was accomplished with a root-canal-shaping rotary nickel-titanium file system (Protaper, Dentsply), after which 10 peptide-treated beads − either with buffer (n = 6), or p-ASARM (n = 6), or np-ASARM (n = 6) − were implanted into the pulp using a blunt steel probe. After bead placement, cavities were filled with Biodentine cement (Septodont, Saint-Maur des Fossés, France). The rats were sacrificed after one month and X-ray micro-computed tomography (micro-CT) analysis was performed on the treated molars (Skyscan Model 1172, Kontich, Belgium). Semi-automatic segmentation of X-ray serial images allows the calculation of the unmineralized pulp volume in order to indirectly quantify the mineralization of the reparative dentin within the teeth. After X-ray scanning, tissue demineralization was then performed in 4.13% EDTA for approximately 8 weeks. After tissue embedding in paraffin (Paraplast), 7-µm-thick sections were cut on a rotary microtome and either prepared for staining with hematoxylin-eosin stain, or prepared for immunohistochemistry. Sections were deparaffinized and rehydrated before blocking with 10% normal goat serum for 45 min at room temperature. Sections were incubated overnight at 4°C with primary antibody against

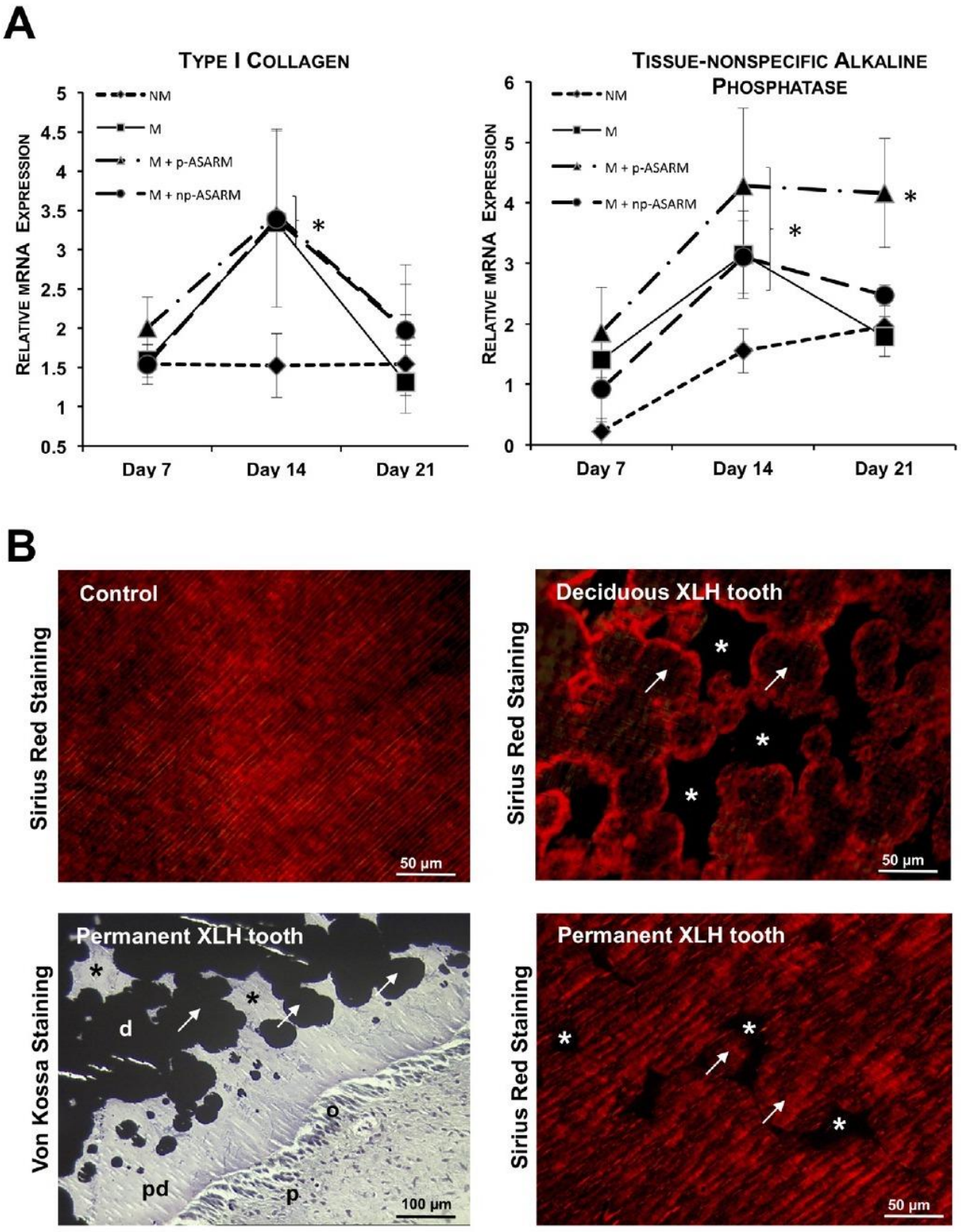

Figure 3. Effect of MEPE-ASARM peptides on type I collagen and tissue-nonspecific alkaline phosphatase expression. A: Quantitative real-time PCR (as described in Materials and Methods) for type I collagen and tissue-nonspecific alkaline phosphatase expression in 3D cultures of SHEDs maintained in nonmineralizing (NM) or mineralizing (M) conditions in the absence or presence of either 20 µM phosphorylated (p-ASARM) or

nonphosphorylated (np-ASARM) ASARM peptide for 21 days. mRNA expression levels were upregulated from day 7 to day 14 in M, M+p-ASARM and M+np-ASARM conditions and, at day 21, returned to a level comparable to that of the NM condition, except for alkaline phosphatase which remained high in the M+p-ASARM condition. $n = 3$, error bars +/− SD, * indicates significant difference ($p<0.05$) relative to NM condition. **B:** Sirius Red staining of collagen in deciduous tooth sections from a 3-year-old male XLH patient (upper right panel), permanent tooth sections from a 15-year-old female XLH patient (bottom right panel) and control patient (upper left panel) revealing an intact collagen distribution only where dentin has mineralized in the form of characteristic calcospherites (arrows), and not in the interglobular spaces (asterisks) in the dentin where mineralization is typically impaired in XLH and where matrix degradation occurs. Sirius Red staining of normal dentin (right panel) is generally homogeneous across the tubular dentin. Similarly, von Kossa staining counterstained with toluidine blue in the same XLH permanent tooth sections (bottom left panel) show lack of fusion of calcospherites (arrows) with large non-mineralized interglobular spaces (asterisks) in the dentin. o: odontoblast. pd: predentin. d: dentin. p: pulp.
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the MEPE mid-region at a 1/200 dilution and processed as described above in the immunohistochemistry section.

Human XLH Tooth Characterization

Patient characteristics are given in Table 2. All teeth were obtained with the informed and oral consent of the patients and the parents, according to ethical guidelines set by the French government (see above). Human deciduous incisors were collected from a 3-year-old male patient with XLH [26]. Immediately after extraction, teeth were fixed for 7 days at 4°C in a 4% paraformaldehyde solution buffered at pH 7.3 by PBS and processed after demineralization [7] for Sirius red staining.

Human permanent third molar germs were collected in the context of an orthodontic treatment plan from a 15-year-old female patient with XLH [26]. Immediately after extraction, teeth were fixed for 7 days at 4°C in 70% ethanol, included in methyl methacrylate [44] and processed for von Kossa and Sirius red staining.

Statistical Analysis

Values are represented as the mean +/− standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Fisher's least significant difference test.

Results

Dose-dependent Response of SHEDs to p-ASARM Peptide

SHEDs were cultured in a 3D collagen matrix enclosed within a tooth slice under mineralizing conditions (Fig. 1A). Cells were treated at concentrations ranging from 5 to 20 μM of phosphorylated MEPE-derived ASARM peptide (p-ASARM) for 21 days in mineralizing medium (Fig. 1B). p-ASARM peptide was shown to inhibit mineralization nodule formation in a dose-dependent manner with the highest inhibition occurring at the highest concentration (20 μM) of p-ASARM, which is in agreement with preliminary data obtained with 2D cultures of either human dental pulp stem cells or SHEDs (data not shown), and with previous studies [33,38]. This 20 μM concentration was subsequently used for all other experiments. In contrast, nonphosphorylated MEPE-derived ASARM peptide (np-ASARM) had no effect on mineralization as previously reported with other cell types [14,33].

p-ASARM Peptide Inhibits ECM Mineralization in a 3D Culture of SHEDs

To examine the role of p-ASARM on ECM mineralization, SHEDs were cultured for 21 days in the 3D model under mineralizing (M) or nonmineralizing (NM) conditions, or under mineralizing conditions with either 20 μM of p-ASARM (M+p-ASARM) or np-ASARM (M+np-ASARM). No mineralization was observed in the presence of p-ASARM, whereas readily distinguishable mineralized nodules were observed in the absence or the presence of np-ASARM (Figs. 1C). Calcium quantification from the cell cultures performed by flame atomic adsorption spectrometry confirmed the mineralized nodule counts by showing very low calcium levels in the M+p-ASARM treatment condition similar to the nonmineralizing condition calcium content (Fig. 1D).

Analysis of samples by scanning electron microscopy (SEM) of fractured collagen/tooth slice scaffolds showed mineralized nodules within the collagenous ECM in close association with neighboring cells (Figs. 2A, B). In all conditions, well-distinguishable cell processes were observed in contact with the tooth slice (Fig. S2). Calcium-to-phosphate ratios of mineralized nodules obtained by EDX spectroscopy were close to 1.67 for the two conditions permissive of mineralization (M, and M+np-ASARM, Fig. 2B), which is consistent with the formation of a hydroxyapatite-like mineral phase. In contrast, cell cultures treated under NM conditions, or with the M+p-ASARM condition, showed no evidence of mineralization (Fig. 2A). Under the conditions where mineral formed, analysis by light microscopy and by TEM of mineralized scaffold matrix regions showed a small nodular type of mineralization amongst the collagen fibrils, with some mineralization extending occasionally along collagen fibrils (Fig. 2C); no such mineralization was observed in cultures treated with p-ASARM (data not shown).

p-ASARM does not Modify Type I Collagen mRNA Expression but Upregulates Tissue-nonspecific Alkaline Phosphatase mRNA

The aim of seeding SHEDs in a 3D collagen matrix was to generate an ECM environment mimicking that found in vivo which favors the cell differentiation process of progenitor/stem cells [48,49,50]. We therefore investigated some of the major ECM components and marker proteins known to be expressed by SHEDs as they begin to differentiate towards the odontoblast phenotype. The number of transcripts encoding type I collagen − the major ECM protein of odontoblasts/dentin − increased under mineralizing conditions from day 7 to day 14 (approximately 2-fold), after which it returned at day 21 to the basal expression level of cells grown under the nonmineralizing condition (Fig. 3A); this induction was not modified by the presence of the ASARM peptides. Likewise, alkaline phosphatase mRNA expression was upregulated in all mineralizing conditions between days 7 and 14 (Fig. 3A). Thereafter, it remained stable up to day 21 in the presence of p-ASARM, but diminished to a level comparable to the nonmineralizing condition in the other conditions (M and M+np-ASARM).

As collagen has never been investigated in teeth from patients with XLH, we examined its distribution in tooth sections. Sirius red staining, which stains collagen fibrils, was associated predominantly with the calcospherites characteristic of this pathology, indicating that there was little or no collagen (degraded) in the interglobular spaces (Fig. 3B) as previously shown [12].

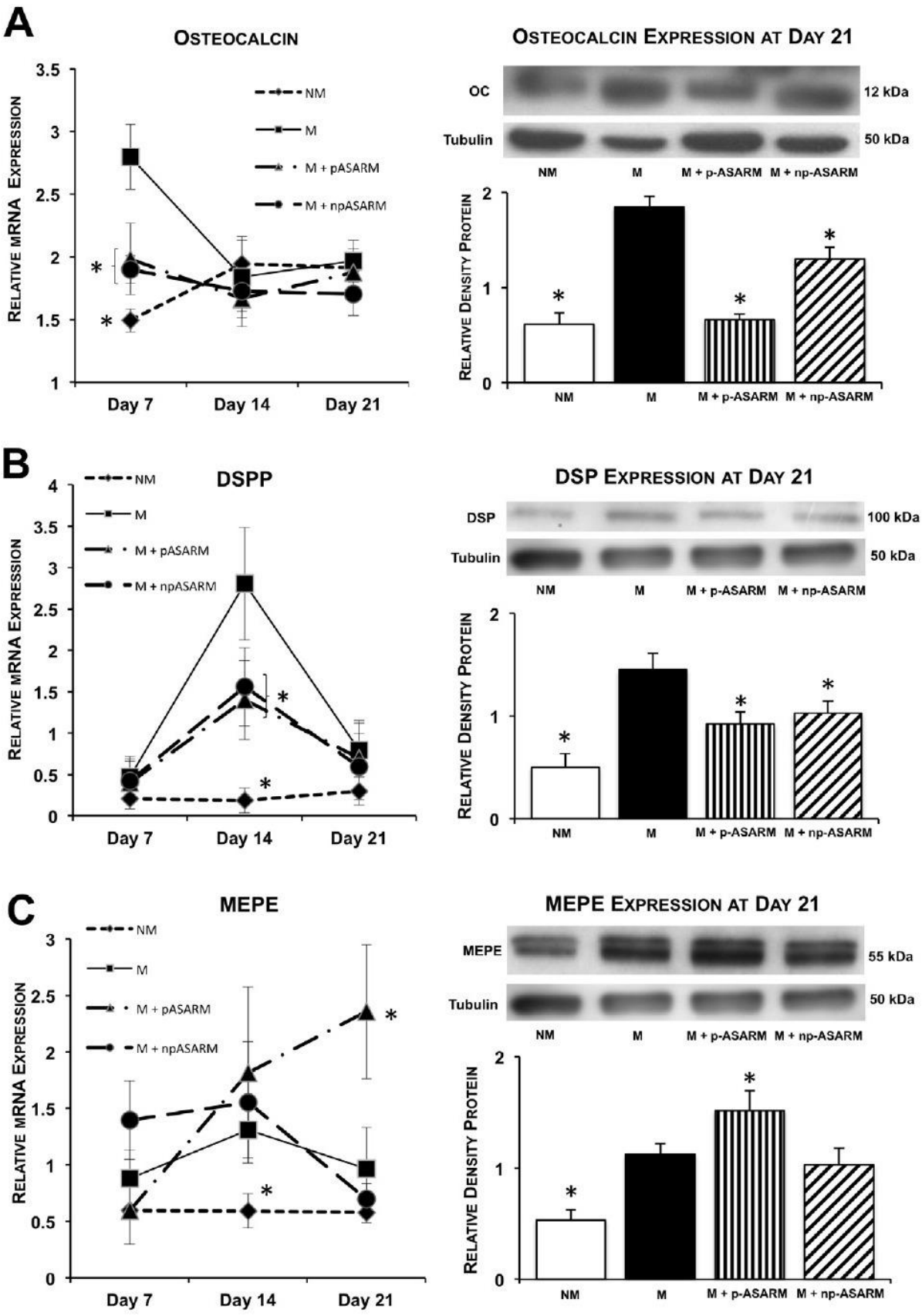
A
OSTEOCALCIN
RELATIVE MRNA EXPRESSION
NM
M
M + pASARM
M + npASARM
Day 7
Day 14
Day 21
OSTEOCALCIN EXPRESSION AT DAY 21
OC
12 kDa
Tubulin
50 kDa
NM
M
M + p-ASARM
M + np-ASARM
RELATIVE DENSITY PROTEIN
B
DSPP
DSP EXPRESSION AT DAY 21
DSP
100 kDa
C
MEPE
MEPE EXPRESSION AT DAY 21
55 kDa

Figure 4. Effect of MEPE-ASARM peptides on osteocalcin, DSPP and MEPE expression. SHED cell cultures were maintained in nonmineralizing (NM) or mineralizing (M) conditions in the absence or presence of 20 μM of either phosphorylated (p-ASARM) or nonphosphorylated (np-ASARM) ASARM peptide for 21 days. Quantitative real-time PCR at day 7, 14 and 21, and Western blotting at day 21, for osteocalcin, DSPP/DSP and MEPE were performed. **A,B:** Osteocalcin and DSPP/DSP expression are induced under the M condition both at the mRNA and protein levels. This induction is reduced in the presence of both the p-ASARM and np-ASARM peptides. **C:** mRNA and Western blot analysis for MEPE reveal increased expression of MEPE in all mineralizing conditions from day 7 to day 14 (expression was not detectable at baseline). At day 21, MEPE expression was still strongly upregulated in the M+p-ASARM condition. $n = 3$, error bars +/− SD, * indicates significant difference ($p<0.05$) relative to the mineralizing condition without peptides (M).
doi:10.1371/journal.pone.0056749.g004

Odontoblast Differentiation is Impaired in the Presence of ASARM Peptides

Differentiation of SHED cells to odontoblasts is associated with an increase in mineralization-regulating protein marker gene expression. Osteocalcin mRNA was increased in all mineralizing conditions at day 7 relative to the nonmineralizing condition, an expression pattern substantially reduced in the presence of the ASARM peptides (Fig. 4A). Western blot analysis confirmed a lower expression of the osteocalcin protein in the presence of the ASARM peptides and most particularly after addition of p-ASARM. Osteocalcin immunolocalization revealed variable levels of cell immunostaining under all conditions. In addition, mineralized nodules in the mineralizing conditions (M and M+np-ASARM) were stained for osteocalcin (Fig. 5, top row, arrows) whereas no ECM labeling was observed in the p-ASARM-treated samples and in the nonmineralizing condition. Analysis of the expression of the dentin sialoprotein protein (DSP) precursor protein DSPP, another marker of odontogenic differentiation [51], revealed an increase in its transcript number at day 14 in all mineralizing conditions as compared to the nonmineralizing condition (Fig. 4B). However, in the presence of the ASARM peptides, this upregulation was twice as low when compared to control mineralizing conditions. Such a differential expression pattern was confirmed at the protein level by Western blot analysis at day 21. For DSP, positive immunohistochemical staining was revealed in the mineralized nodules in the ECM (Fig. 5, middle row, arrows in M and M+np-ASARM conditions).

p-ASARM Peptide Upregulates MEPE Expression

Given the mineralization-inhibiting properties of the MEPE-derived p-ASARM peptide and also its effects on expression of odontoblast marker proteins, we next examined the expression of *MEPE* itself after the 3D cultures were treated with the ASARM peptides. As presented in Figure 4C, MEPE transcript expression was induced from day 7 to day 14 in all mineralizing conditions, regardless of the presence of the ASARM peptides. However, at

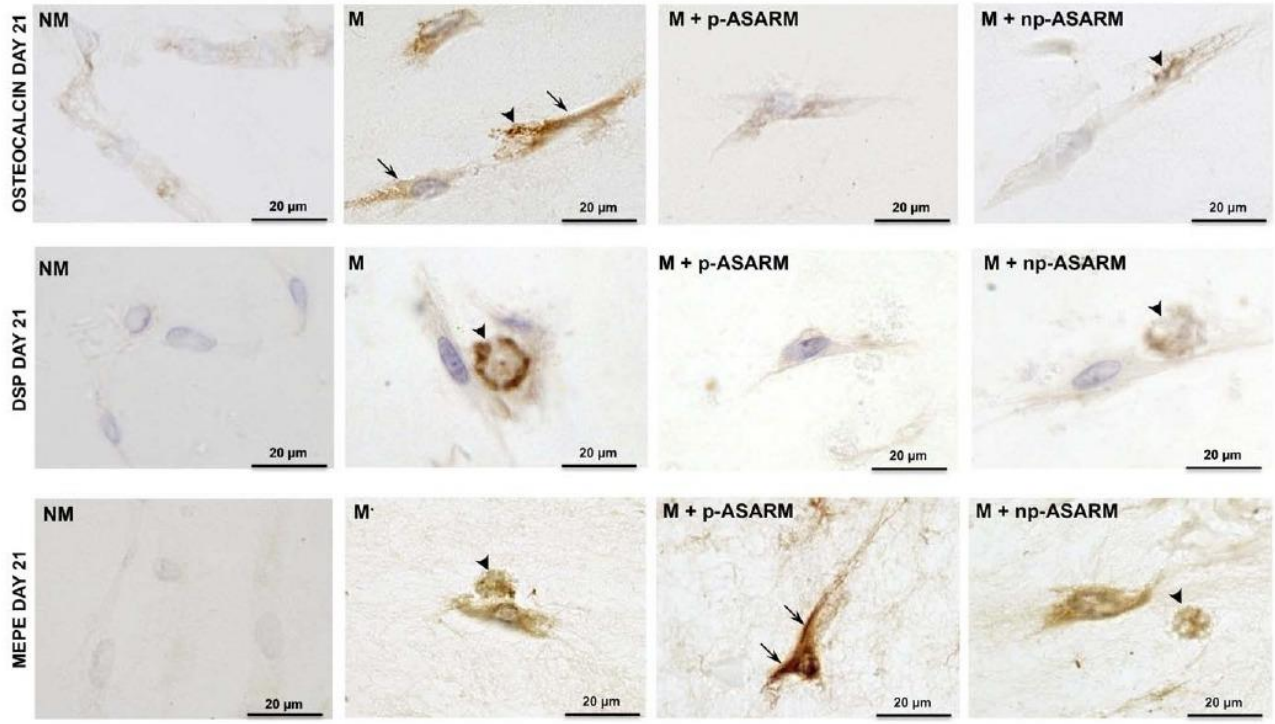

Figure 5. Immunohistochemical detection of osteocalcin, DSP and MEPE after treatment with MEPE-ASARM peptides. SHED cell cultures were maintained in nonmineralizing (NM) or mineralizing (M) conditions in the absence or presence of 20 μM of either phosphorylated (p-ASARM) or nonphosphorylated (np-ASARM) ASARM peptide for 21 days. Immunohistochemistry for osteocalcin, DSP and MEPE was performed on methyl methacrylate sections of 21-day cultures. Osteocalcin immunostaining (top row) is strong in SHEDs (arrows) and nodules (arrowheads) in the M and M+p-ASARM conditions. Immunohistochemistry for DSP shows strong staining in mineralized nodules in both the M and M+np-ASARM conditions. MEPE immunostaining is found in SHEDs and nodules in mineralizing conditions the (M and M+np-ASARM conditions) but is particularly strong in the cultures treated with p-ASARM that do not mineralize (M+p-ASARM). The images presented are representative of all sections examined.
doi:10.1371/journal.pone.0056749.g005

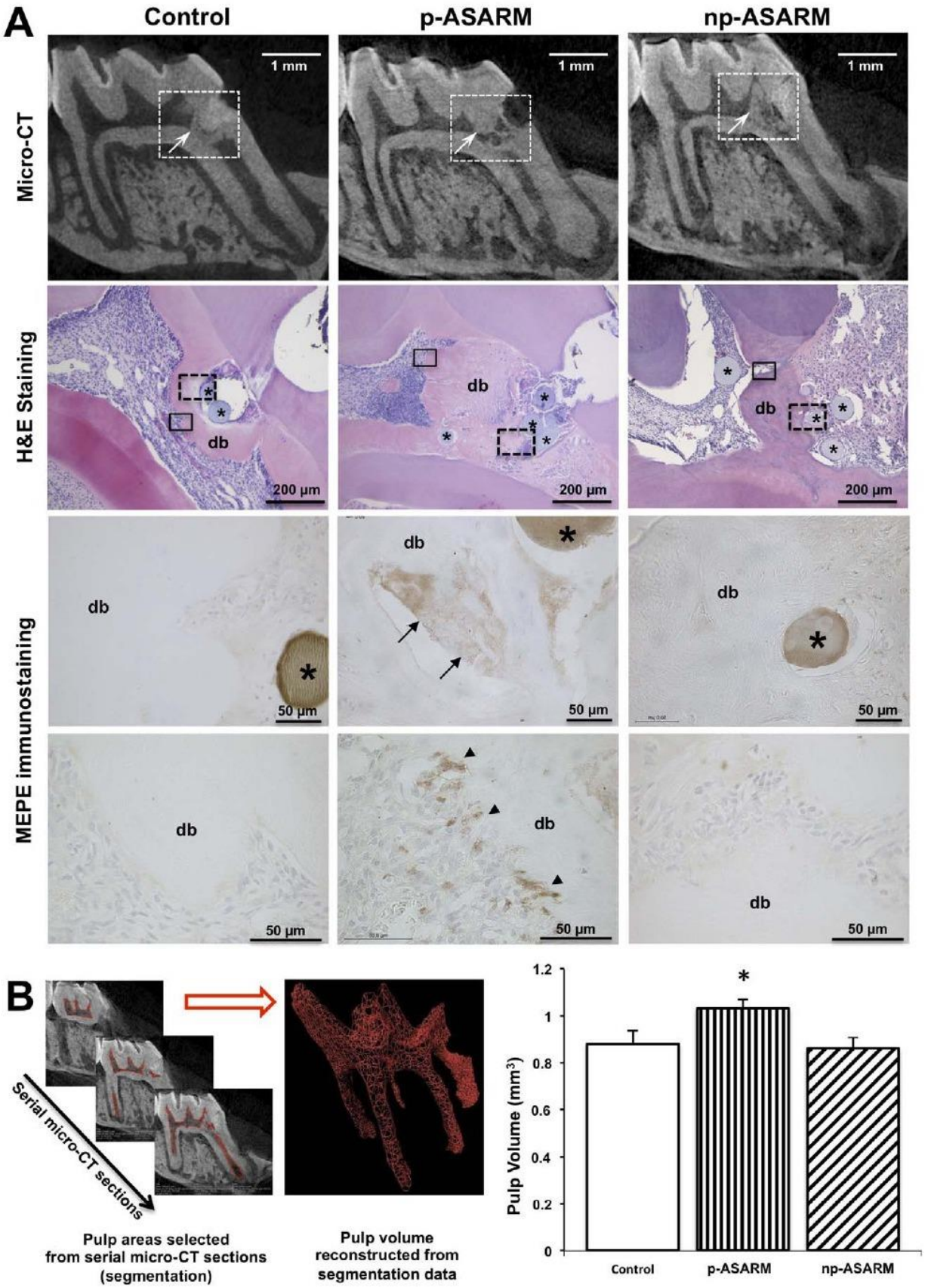
A
Control
p-ASARM
np-ASARM
Micro-CT
H&E Staining
MEPE immunostaining
1 mm
200 µm
50 µm
db
B
Serial micro-CT sections
Pulp areas selected from serial micro-CT sections (segmentation)
Pulp volume reconstructed from segmentation data
Pulp Volume (mm³)
1.2
1
0.8
0.6
0.4
0.2
0
Control
p-ASARM
np-ASARM

Figure 6. Effect of MEPE-ASARM peptides on reparative dentin mineralization and MEPE expression in a rat dentin/pulp wound model. This *in vivo* data is from rats sacrificed at 1 month after implantation of agarose beads soaked in either p-ASARM or np-ASARM, or buffer (control), into the pulp of the first upper molar. A: MEPE derived p-ASARM impairs reparative dentin mineralization and enhances MEPE expression. Micro-computed tomography (micro-CT) sections (top row) show mineralization (arrows) occurring in the pulp chamber as a reparative process (dentin bridge formation) near the pulp injury (white dashed box outline). The dentin bridge appears thinner and irregular with large voids (mineralization defects) in the p-ASARM treatment when compared to the control and np-ASARM treatment. Hematoxylin and eosin (H&E) staining of paraffin histology sections (second row) taken from the region outlined by the white dashed boxes show the irregular nature of the dentin bridge (db) in the vicinity of the beads (asterisks) soaked in p-ASARM as compared to the control and the np-ASARM treated wounds. Immunohistochemistry for MEPE (third row) in the region demarcated by the black dashed box outlines shows an accumulation of MEPE (arrows) in nonmineralized spaces of the reparative dentin bridge only in the case p-ASARM treatment. Immunohistochemistry for MEPE (bottom row) in the region demarcated by the black box outlines shows strong MEPE staining of cells (odontoblasts) secreting the dentin bridge. In control and np-ASARM-treated samples, the dentin bridge appears homogenous and with no or weak staining for MEPE. B: Quantification of the pulp volume (to indirectly measure mineralization volume "pulp fill") was performed by micro-CT of the bead-implanted molars. Semi-automatic segmentation (red area) of the pulp was performed from micro-CT serial sections in order to calculate the global volume of the pulp. The volume of the pulp from the p-ASARM-treated molars was significantly higher when compared to the control and np-ASARM treatment suggesting that mineralization in the reparative dentin process was impaired by p-ASARM. * indicates significant difference ($p<0.05$) relative to control.
doi:10.1371/journal.pone.0056749.g006

day 21, MEPE transcripts remained significantly increased (2.8-fold) in the presence of p-ASARM peptide, whereas they returned to levels in the other mineralizing conditions (M and M+np-ASARM) comparable to those observed in nonmineralizing conditions. This result was confirmed by Western blot analysis where MEPE protein expression increased in all mineralizing conditions (Fig. 4C), but its expression was significantly higher in the presence of p-ASARM. Consistent with this, immunohistochemistry for MEPE showed particularly strong cell staining for the p-ASARM treatment under mineralizing conditions (Fig. 5, bottom row, arrowheads). In contrast, in the presence of np-ASARM or in the mineralizing condition alone without added peptide, the staining was associated with the mineralized nodules in the ECM, with moderate staining of the cells (Fig. 5, bottom row, arrows). These results indicate that the p-ASARM peptide increases MEPE expression by differentiating SHEDs via a positive feedback mechanism. Given that MEPE has protein-protein interactions with PHEX, and given that its ASARM peptide is a substrate for PHEX enzymatic activity, we examined PHEX expression *in vitro* under our different treatment conditions applied to the SHED cells in 3D cell culture. In this collagen/tooth slice scaffold model, PHEX expression was not altered by ASARM treatment (Fig. S3).

p-ASARM Peptide Impairs Dentin Formation in a Rat Model of Pulp Repair

To evaluate whether ASARM peptides display similar properties on dentin mineralization in teeth *in vivo*, we implanted agarose beads soaked in either p-ASARM or np-ASARM peptide, or control buffer, into injured dental pulp in a tooth-wound model in young rats. In this model, the repair process is mediated by endogenous pulp cells undergoing odontoblast differentiation [47]. Micro-CT analysis and histology after hematoxylin and eosin staining at 1 month following surgery and peptide implantation showed a reparative mineralized dentin bridge in all samples (Fig. 6A); however, it appeared thicker in both control and np-ASARM treated pulps when compared to the p-ASARM treated teeth. To quantify this reparative process using micro-CT, the pulp volume was segmented in order to calculate its global volume (Fig. 6B). The pulp volume was significantly greater in the p-ASARM-treated samples (1.03 mm^3+/−0.04) (thus less mineralized dentin bridge tissue) than in both the control and np-ASARM conditions (0.88 mm^3+/−0.06 and 0.86 mm^3+/−0.05, respectively). These results in an animal model are consistent with our other *in vitro* SHED cell culture observations and show that the p-ASARM peptide impairs dentin neo-formation and its mineralization after pulp injury. Given our *in vitro* data indicating that mineralization inhibited by p-ASARM was accompanied by an increased expression of MEPE, we then evaluated MEPE expression in this *in vivo* model using an antibody directed against the mid-region of MEPE. Nonmineralized spaces within a heterogeneous dentin bridge stained positively for MEPE in the p-ASARM treated samples (Fig. 6A). In addition, cells adjacent to and likely secreting this dentin bridge were strongly stained for MEPE. In contrast, in control and np-ASARM samples, a homogenous reparative bridge was seen with no MEPE staining. Taken together, these results demonstrate that the p-ASARM peptide enhances MEPE expression by differentiating dental pulp stem cells both *in vivo* and *in vitro*.

Discussion

A MEPE-derived ASARM peptide was previously identified in human hypophosphatemic tooth dentin [12]. Together with the results of the present study, these data present evidence that this peptide (and potentially other similar ASARM peptides derived from other matrix SIBLING proteins) may be involved in impairing tooth dentin mineralization as observed in patients affected with XLH. Using a cell culture collagen/tooth slice 3D scaffold model, together with an *in vivo* wound healing animal model, the present study demonstrates that the MEPE-derived ASARM peptide inhibits odontogenic differentiation of deciduous dental pulp stem cells (SHEDs), enhances MEPE expression, and impairs dentin ECM mineralization.

Cell functions such as migration, adhesion and differentiation are routinely studied in 2D cultures. However, interpretations from these *in vitro* models are limited by the fact that they do not accurately recreate the appropriate, natural tissue architecture [48,52,53,54,55]. We have therefore developed and used a 3D collagen/tooth slice scaffold model where seeded cells can undergo differentiation in a more natural ECM environment [41], as demonstrated by the expression of several well-known odontoblast markers commonly used to identify this cell type (without the added tooth slice, no odontoblast marker expression was observed – data not shown). Based on our previous clinical and histologic findings [3,6] together with observations made by others [4,8] reporting severe abnormalities in deciduous teeth of patients with XLH (spontaneous dental abscesses associated with enamel cracks, poorly mineralized dentin, large pulp chamber and prominent pulp horns), we used 3D culture of dental pulp cells which displayed a typical SHED phenotype [56] as confirmed by polychromatic flow cytometry (Fig. S1), and a tooth/dentin wounding model in rats to examine further the causes of XLH tooth pathology, with a focus on the effects of the MEPE ASARM peptide. Under mineralizing culture conditions without added peptide, we observed von Kossa-stained mineralized nodules in

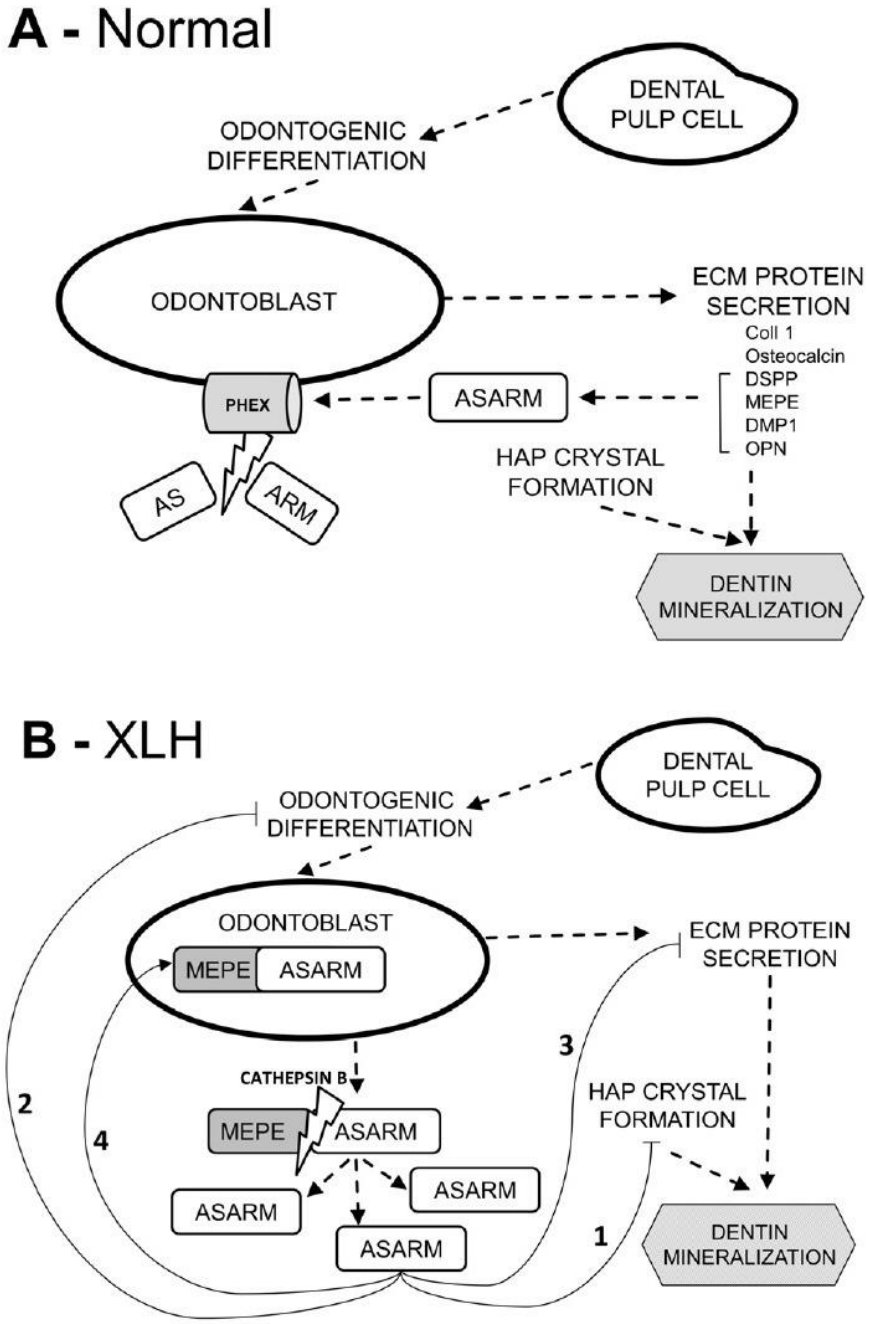

Figure 7. Summary of the role of the MEPE-derived ASARM peptide in the etiology of tooth dentin abnormalities in XLH patients. A: SIBLING proteins containing the ASARM peptide are processed by a multitude of proteolytic enzymes, some of which may release the ASARM peptide or larger protein fragments containing the ASARM peptide into the extracellular matrix (ECM). ASARM and ASARM-containing peptides are inhibitory for mineralization, binding directly to hydroxyapatite (HAP) mineral crystals in bones and teeth. In normal conditions, neutralizing PHEX cleavage of ASARM releases extracellular matrices from this inhibition and mineralization proceeds appropriately. B: In XLH tooth dentin, inactivating mutations in the *PHEX* gene result in nonfunctional PHEX enzyme that allows HAP crystal-binding, ASARM-containing peptides to accumulate in the dentin thus inhibiting tooth mineralization (pathway 1). Normal PHEX also protects full-length MEPE from cleavage by proteases (cathepsin B), thereby preventing release of mineralization-inhibiting ASARM. In XLH, excessive cleavage of MEPE by proteases (in the absence of functional PHEX) to release the inhibitory ASARM peptide might also contribute to the impaired mineralization of dentin. Finally, ASARM accumulation in XLH may impair dentinogenesis by decreasing odontoblast differentiation and downregulating genes encoding for secreted ECM proteins (pathways 2 and 3), while increasing MEPE expression (pathway 4) which in turn would further exacerbate the XLH hypomineralization tooth phenotype.
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the ECM which were in close proximity to the cells and not more distal in the collagen scaffold, suggesting that they resulted from a cell-mediated biomineralization process rather than from random (dystrophic) mineral deposition within the collagen scaffold. Collagen mineralization along collagen fibrils was confirmed morphologically by SEM and TEM analyses and by microana-

lytical EDX demonstrating high calcium and phosphorus levels within the nodules with calcium/phosphate ratios being comparable to those observed in human mineralized bone and tooth extracellular matrices [57,58,59. In addition, cells differentiated from normal SHEDs expressed a variety of mineralization-relevant biomarkers including PHEX, which demonstrates the utility of our collagen scaffold *in vitro* model in providing a suitable 3D matrix environment enabling odontoblast cell differentiation. Taken together, these observations confirm that the culture model used here was appropriate to evaluate SHED cell differentiation in response to addition of p-ASARM – a circumstance thought to be relevant to bone and tooth mineralization in the pathologic XLH condition where available evidence describes an accumulation of ASARM-containing (and other) peptides derived from the SIBLING proteins [14,32,33,36,37,60]. Whereas most of the studies reported to date describe the effects of ASARM peptides and their degradation by PHEX on mineralization in bone cell cultures, the present study is the first to extend these results to mineralization in odontoblast cultures, and to describe effects of ASARM on cell differentiation.

In terms of its effects on mineralization in the SHED/odontoblast 3D cultures, the phosphorylated form of the MEPE-derived ASARM peptide (p-ASARM) inhibits the formation of mineralization nodules in the collagenous ECM, whereas the non-phosphorylated form has no effect on mineralization. This observation is consistent with previous findings from bone cell cultures showing that the phosphorylation of MEPE- and OPN-derived ASARM peptides is necessary for the inhibition of ECM mineralization [14,32,33,37,60]. These previous bone cell studies demonstrated that the ASARM peptides inhibit mineralization in a phosphorylation-dependent manner via direct binding to hydroxyapatite crystals [14,33]. Interestingly, the p-ASARM dosage used by us and others to inhibit mineralization in cell culture (20 μM) is similar to the mean levels of ASARM peptide directly measured in the serum of patients with XLH, and in the serum of homolog Hyp mice (16 and 23 μM, respectively) [61].

In terms of its effects on cell differentiation in the SHED 3D cultures, we have made here the additional finding that ASARM peptide impairs dental pulp stem cell differentiation into odontoblast-like cells, an important observation with direct relevance to the pathologic mechanisms underlying XLH. The modified expression of odontoblast markers (DSPP, osteocalcin, alkaline phosphatase and MEPE) in the presence of ASARM peptides is a novel finding for tooth cells, and we further show that such an influence is more pronounced after exposure to the phosphorylated form of the peptide. Such observations are consistent with a previous study conducted on murine bone marrow stromal cells differentiating into osteoblasts, which reports a modification of osteoblast gene expression in the presence of MEPE-derived ASARM peptide [14]. Contrary to this, another study reported no effect of ASARM on cell differentiation using the murine MC3T3-E1 pre-osteoblast cell line [33]. This discrepancy may be explained by differences in cell characteristics (*e.g.* stem cells versus partially committed cells) and by the fact that these previous studies were carried out in 2D cell cultures.

In the present study, odontoblast differentiation was substantially impaired in the presence of p-ASARM as evidenced by the decrease (both at the mRNA and protein levels) of markers related to the odontoblast program such as DSPP and osteocalcin, whereas there was only a slight effect on these markers when np-ASARM was added.

The decreased osteocalcin expression reported here is consistent with those previously reported in bone cell cultures treated with p-ASARM [14], and also with our report on dentin matrix proteins in XLH deciduous human teeth where we showed by Western blot analysis of dentin extracts a decrease in osteocalcin and DSP compared to age-matched controls [7]. In contrast, type I collagen, the most prominent protein of dentin ECM, was not modified by the addition of the ASARM peptides in our present cell culture study. Indeed, type I collagen expression followed a pattern comparable to that previously reported for osteogenic cells cultured in a collagenous ECM-like 3D scaffold and treated with ascorbic acid [53]. In hypomineralized XLH human dentin, it appears that ECM matrix components are degraded at sites where the dentin does not mineralize [7].

Expression of tissue-nonspecific alkaline phosphatase (ALPL, TNAP) was up-regulated in our experiments in all mineralizing conditions from day 7 to day 14. This phosphatase catalyzes the hydrolysis of most phosphomonoesters – particularly cleavage of the potent mineralization-inhibiting small molecule pyrophosphate (PPi) found in serum, most tissue fluids and ECMs – releasing inorganic phosphate (Pi) which could contribute to local phosphate levels and mineralization [62]. In the present study, mRNA levels of alkaline phosphatase were upregulated prior to ECM mineralization and downregulated when mineralized nodules became apparent by bright field light microscopy after von Kossa staining. This expression pattern has been previously reported during the differentiation of human and murine osteogenic cell lines cultured within a dense collagen scaffold [53,63]. We additionally found that in the presence of the p-ASARM peptide, alkaline phosphatase mRNA levels remained elevated at the later culture time point (day 21). Such a difference might be explained by the fact that although most of the required conditions for mineralization *in vitro* have been met and are present in the cultures – including high levels of phosphate and calcium for mineralization, ascorbic acid for synthesis and secretion and assembly of collagen fibrils, and noncollagenous protein secretion – mineralization has not occurred in the presence of p-ASARM, and the cells are sensing that excess inhibitory PPi may need to be removed by continuing their ALPL expression. Interestingly, and similar to this observation, the serum of patients with XLH shows increased alkaline phosphatase activity, being especially high in children prior to their treatment (with vitamin D analogues and phosphate supplements) [64]. For example, in a group of 28 children affected with XLH and having a PHEX mutation, with a mean age of 2.2±1.1 years, the mean ± SE alkaline phosphatase activity before any treatment was 1,320±910 IU/L, being more than 4 times higher than the upper normal range (normal range is inferior to 300 IU/L); data from Centre de Référence des Maladies Rares du Métabolisme du Phosphore et du Calcium, AP-HP, Kremlin Bicêtre, France).

Our *in vivo* observations using a rat pulp injury model also support an inhibitory effect of phosphorylated MEPE-derived ASARM peptide on the formation of mineralized dentin. In this model, a reparative dentin bridge normally forms following injury and operates as a barrier to protect the dental pulp [39,47]. We previously reported abnormal dentin mineralization in the proximity of p-ASARM peptide soaked beads using this rat model [12]. Here we show in additional studies that only the phosphorylated form of the ASARM peptide impairs mineralization, and that immunohistochemistry for MEPE shows more intense staining of odontoblasts adjacent to the reparative dentin bridge after p-ASARM treatment than after np-ASARM treatment as applied by peptide-soaked agarose bead placement at the wound site. Moreover, here we have quantified pulp volume to show that it is significantly higher in rat molars treated with inhibitory p-ASARM, indicating decreased mineralization relative to controls. Paralleling this, when compared to age-matched

control patients with normal teeth, we previously reported a 2-fold higher pulp ratio (pulp chamber area/tooth area) in deciduous molars of children with XLH, and a 1.6-fold higher pulp ratio in permanent teeth of young adults with XLH [3].

The cleavage and protein-protein interactions of MEPE have been implicated in the pathogenicity of XLH [23]. Notably, it has been reported that MEPE is downregulated as odontoblasts differentiate [65,66]. In our *in vitro* experiments, p-ASARM significantly increased MEPE expression, both at the mRNA and protein levels. In addition, odontoblast-like cells producing reparative bridge dentin in rat dental pulps implanted with p-ASARM peptides strongly expressed MEPE. Thus, our *in vitro* and *in vivo* results indicate that the p-ASARM peptide enhances MEPE expression, which in turn might delay pulp stem cell differentiation as might be expected to occur in XLH patients. Moreover, accumulation of MEPE in the unmineralized heterogeneous dentin bridge regions identified in p-ASARM-treated rats is consistent with our previous observation showing that MEPE accumulates in the unmineralized interglobular spaces (between calcospherites) of dentin in patients with XLH [13]. Furthermore, the increased expression of MEPE we observed in the presence of p-ASARM both in the 3D culture model and in the wounded pulp rat model is consistent with previous publications reporting an upregulation of MEPE in osteoblasts of Hyp mice [16,20,23,37]. Thus, in XLH, overexpressed MEPE in osteoblasts (and shown here now in odontoblasts) results in the potential for more ASARM peptide to accumulate in the ECM of bone and dentin, which would not be cleaved away (as would normally occur by the enzymatic activity of PHEX) as a result of the inactivating PHEX mutations (summarized in Fig. 7). As the ASARM peptides are highly resistant to proteases and are only known to be degraded by PHEX [23,33], this positive-feedback loop on MEPE expression induced by ASARM peptides might exacerbate the disease and would thus be a mechanism contributing in part to the mineralization defect seen in XLH. Indeed, increased levels of ASARM peptide in serum have been reported in hypophosphatemic patients and in Hyp mice [31,61]. Furthermore, the ASARM peptide has been shown to accumulate in the renal tubules of Hyp mice [61], and recent evidence suggests that it may directly impair phosphate uptake and induce hypophosphatemia [31]. Studies are currently in progress using pulp cells derived from XLH patients to better understand the function of PHEX in this complex pathologic process.

In conclusion, using *in vitro* and *in vivo* models we demonstrate that the MEPE-derived ASARM peptide – previously identified in XLH dentin – inhibits both ECM mineralization and odontoblast differentiation, while increasing MEPE expression. These results provide for a partial mechanistic explanation of the pathogenesis of XLH whereby direct inhibition of mineralization by p-ASARM positively reinforced by increased MEPE expression induced by ASARM, together with decreased odontoblast differentiation to produce less dentin, leads to the mineralization defect and increased pulp chamber characteristic of teeth in XLH. Further studies are required to determine whether the MEPE-derived ASARM peptide affects odontoblast differentiation through indirect cell-signaling induced by defective ECM mineralization and/or by direct cell signaling with potential internalization of the peptide by cells. Collectively, these data appear promising in guiding the development of novel therapies for XLH through targeting the MEPE-ASARM system.

Supporting Information

Figure S1 Polychromatic flow cytometry analysis of dental pulp cells from deciduous teeth. More than 95% of cells at passage 2 were CD45-. In addition, most of them were CD90+/CD29+/CD44+/CD146+/CD105+/CD73+. About 10% were STRO-1+.
(TIFF)

Figure S2 Interaction of cells with the tooth dentin slice. Scanning electron microscopy of an interaction of SHEDs with the tooth dentin slice at day 21. Images show a close relationship between the cell processes (asterisks) and the dentin surface (containing many dentinal tubules). Higher magnification of the white dashed frame is shown in the right panel.
(TIFF)

Figure S3 MEPE-ASARM peptides do not affect PHEX expression. SHED cell cultures were maintained in nonmineralizing (NM) or mineralizing (M) conditions in the absence or presence of 20 μM of either phosphorylated (p-ASARM) or nonphosphorylated (np-ASARM) ASARM peptide for 21 days. Immunofluorescence microscopy (left panel) and Western blotting (right panel) were performed at day 21. Immunofluorescent staining for PHEX (arrows) is observed in the SHEDs under all conditions. Western blot analysis shows similar levels of PHEX protein without any significant differences between the different conditions, when normalized to cellular tubulin content.
(TIFF)

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Author Contributions

Conceived and designed the experiments: BS CB SOV CC. Performed the experiments: BS CB MK JN BRC BB FL JL FD DLD AN. Analyzed the data: BS EH AL MDM CC AN. Contributed reagents/materials/analysis tools: FL AP PSR AL DLD. Wrote the paper: CC BS EH AP AL MDM.

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Abstract

Role of EMMPRIN and MMPs in tooth development, dental caries and pulp-dentin regeneration

Tooth development is regulated by a series of reciprocal inductive signalings between the dental epithelium and mesenchyme, which culminates with the formation of dentin and enamel. EMMPRIN/CD147 is an Extracellular Matrix MetalloPRoteinase (MMP) INducer that mediates epithelial-mesenchymal interactions in cancer and other pathological processes and is expressed in developing teeth.

Here we used EMMPRIN knockout (KO) mice to determine the functional role of EMMPRIN on dental tissues formation. We demonstrated that EMMPRIN deficiency results in decreased in MMP-3 and MMP-20 expressions, delayed in basement membrane degradation in tooth germ, delayed in enamel formation well distinguishable in incisor, and in decreased enamel volume and thickness but normal maturation. These results indicate that EMMPRIN is involved in the epithelial-mesenchymal cross-talk during tooth development by regulating the expression of MMPs.

Then we tried to investigate the potential role of EMMPRIN in the pulp dentin repair process by comparing the healing of injured pulps of EMMPRIN KO and WT mice.

Finally, we evaluated the capacity of grape-seed extracts (known to be natural inhibitors of MMPs and used in new daily mouthrinse) to prevent the degradation of human demineralized dentin matrix by MMP-3.

KEY WORDS: TOOTH FORMATION, MMPS, CELL INTERACTION, ENAMEL PROTEINS

Résumé

Rôle d'EMMPRIN et MMPS dans le développement dentaire, la carie dentaire et la régénération pulpo-dentinaire

Le développement dentaire est orchestré par une série de signalisations inductives réciproques entre l'épithélium dentaire et le mésenchyme, qui conduit à la formation de la dentine et de l'émail. EMMPRIN/CD147 est un INducteur des MetalloPRoteinases de la Matrice Extracellulaire (MMPs) qui régule les interactions épithélio-mésenchymateuses dans le cancer et d'autres processus pathologiques et est exprimé lors du développement dentaire.

Ainsi, nous avons utilisé des souris KO pour EMMPRIN pour déterminer le rôle d'EMMPRIN dans la formation des tissus dentaires. Nous avons démontré que l'absence d'EMMPRIN conduisait dans le germe dentaire à une diminution de l'expression de MMP-3 et de MMP-20, à un retard de la dégradation de la membrane basale, à un retard de la formation de l'émail bien visible dans l'incisive à croissance continue, à une diminution du volume et de l'épaisseur d'émail, mais à une maturation amélaire normale. Ces résultats indiquent qu'EMMPRIN est impliqué dans le dialogue épithélio-mésenchymateuse pendant le développement dentaire, principalement par la régulation de l'expression de certaines MMPS.

Nous avons ensuite essayé d'évaluer le rôle potentiel d'EMMPRIN dans le processus de réparation dentaire en comparant la cicatrisation de blessures pulpaires des souris KO pour EMMPRIN à des souris WT.

Enfin, dans un souci de transfert vers la clinique, nous avons évalué la capacité d'extraits de pépin de raisin (connu pour être des inhibiteurs naturels de MMPs) à empêcher la dégradation de la matrice dentinaire humaine déminéralisée et traitée par MMP-3.

MOTS CLÉS: FORMATION DE LA DENT, MMPS, INTERACTION CELLULAIRE, PROTÉINES DE L'ÉMAIL.

Abstract

Role of EMMPRIN and MMPs in tooth development, dental caries and pulp-dentin regeneration

Tooth development is regulated by a series of reciprocal inductive signalings between the dental epithelium and mesenchyme, which culminates with the formation of dentin and enamel. EMMPRIN/CD147 is an Extracellular Matrix MetalloPRoteinase (MMP) INducer that mediates epithelial-mesenchymal interactions in cancer and other pathological processes and is expressed in developing teeth.

Here we used EMMPRIN knockout (KO) mice to determine the functional role of EMMPRIN on dental tissues formation. We demonstrated that EMMPRIN deficiency results in decreased in MMP-3 and MMP-20 expressions, delayed in basement membrane degradation in tooth germ, delayed in enamel formation well distinguishable in incisor, and in decreased enamel volume and thickness but normal maturation. These results indicate that EMMPRIN is involved in the epithelial-mesenchymal cross-talk during tooth development by regulating the expression of MMPs.

Then we tried to investigate the potential role of EMMPRIN in the pulp dentin repair process by comparing the healing of injured pulps of EMMPRIN KO and WT mice.

Finally, we evaluated the capacity of grape-seed extracts (known to be natural inhibitors of MMPs and used in new daily mouthrinse) to prevent the degradation of human demineralized dentin matrix by MMP-3.

KEY WORDS: TOOTH FORMATION, MMPS, CELL INTERACTION, ENAMEL PROTEINS

Résumé

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